The Role of CBP and p300 in Alzheimer's Disease

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"The cure for boredom is curiosity. There is no cure for curiosity."

Dorothy Parker

To date, one IDF soldier is in captivity. This thesis is dedicated to his health and safe return.

Abstract

Studies of the mechanisms underlying memory formation have defined central roles for CRE-dependent gene expression, which is mediated by the transcription factor CREB and the coactivator CBP. CBP creates a bridge between CREB and the basal transcriptional machinery and acetylates histones, which induces chromosomal changes and results in loss of chromosomal repression. This allows successful transcription of the underlying genes needed for synthesis of proteins underlying memory formation. CBP has been linked to neurodegenerative diseases and cerebral CBP levels were shown to reduce in mice lacking functional presenilins (PSs), a class of enzymes that has been associated with Alzheimer's Disease (AD).

In this thesis it is shown that WT PS1 stimulates the transcriptional activating ability of CBP and its close homolog p300, whereas an Alzheimer's disease-associated N terminal mutant of PS1 did not produce this effect. Interestingly, PS1 C terminal mutants produced a reduction in CBP transcriptional activating ability, compared to control levels.

Additionally, we showed that wild type PS1 increases the endogenous CBP level. Moreover, an increase in CBP endogenous levels was noted when the cells were transfected with the -M146L N-terminal mutant of PSI. However, these levels were still significantly lower when compared to cells transfected with wild type PS1. We were also able to show that knockdown of endogenous PS1 leads to a decrease in endogenous CBP levels and a decrease in CBP activity. Hence, PSI can affect both the level and the activity of CBP.

In addition, the activation of CBP by WT PS1 involves the PI 3-kinase, p38 MAP kinase and p42/p44 MAP kinase pathways and targets primarily the C terminus of CBP. It is also shown that the effect of wild-type PS1 is dependent on the histone acetyltransferase activity (HAT) of CBP. Moreover, it was demonstrated that WT PS1, but not its M146L mutant, could increase the promoter activity of c-fos, a CBP HAT dependent target gene.

Additionally, we showed that application of the histone deacetylase inhibitor, TSA, rescued the long-term potentiation and long-term memory defects shown by APP/PS1 mutant mice. Moreover, it was shown that the acetylation level of histone H4 in APP/PS1 mice is lower than that of WT littermates and that TSA injection restores the acetylation of these histones. This is the first study to identify AD as a disease of epigenetic etiology and suggests that enhancing histone acetylation may have potential for the treatment of AD.

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Declaration

All the work presented in this thesis is the work of Yitshak Itsik Francis. Contributions by other researchers are acknowledged in the text.

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Articles published during the course of this work

1. Y.I. Francis, A. Stephanou, D.S. Latchman, CREB-binding protein activation by presenilin 1 but not by its M146L mutant, Neuroreport 17 (2006) 917–921.

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Table of Contents

List of Figures

List of Tables

Abbreviations

AMPA	Amino-3-hydroxy-5-methyl-4-isoxazolepropionic-acid
aa	Amino acid
AD	Alzheimer's disease
APLPs	Amyloid precursor-like proteins
APP	Amyloid precursor protein
Aβ	β-amyloid
bp	Base pairs
BSA	Bovine serum albumin
BST	Basal synaptic transmission
C/EBP	CCAAT/enhancer-binding protein
CaMKII	Calmodulin-dependent Protein Kinase II
cAMP	Cyclic adenosine monophosphate
CBP	CREB Binding Protein
CRE	cAMP response element
CREB	cAMP response element binding protein
DNMTs	DNA methyltransferases
EcD	Ecdysone
ERK	Extracellular signal-regulated protein kinase
FAD	Familial Alzheimer's disease
FBS	Fetal bovine serum
GluRs	Ionotropic glutamate receptors
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HTH	Helix-turn-helix

LB	Luria Bertani
LTM	Long-term memory
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
Neurofibrillary tangles	NFTs
NF-IL6	Nuclear Factor Interleukin 6b
NFTs	Neurofibrillary tangles
NICD	Notch intracellular cytoplasmic domain
NMDA	N-methyl-D-asparate
P/CAF	p300/CBP associated factor
PKA	Protein kinase A
PKC	Protein kinase C
PKC	Protein kinase C
PS	Presenilin
SAHA	Suberoylanilide hydroxamic acid
STAT3	Signal transducer and activator of transcription 3
Tg	Transgenic
ZnFs	Zinc fingers

Chapter 1

1 INTRODUCTION

Cognition can be defined as the acquisition, storing and processing of information in order to be retrieved by the brain. Thus, it is not surprising that cognition relies on our ability to form and retrieve long lasting memories, which allows us to recognize our friends, obtain a language, learn and think. In essence, memory is what allows us to define who we are through personal memories and experiences, which distinguishes us from one another.

The nobel laureate Eric Kandel described the process of memory storage as a dialogue between genes and synapses(Kandel, 2001). Kandel and other researchers were able to show that the formation of long-term memory (LTM) is dependent upon DNA converstion into RNA (transcription)(Dash et al., 1990), synthesis of new protein(Montarolo et al., 1986) and structural change of the synapse(Bailey and Chen, 1983). In addition, work from several laboratories showed that the proper regulation of gene expression in LTM is not only controlled by the transcriptional machinery but also modulated by epigenetics. Epigenetics is defined as the mechanism that changes gene expression by 'marking' DNA or its associated proteins, through processes such as DNA methylation and histone modification, without changing the DNA sequence itself(Rakyan et al., 2001). Epigenetic mechanisms are increasingly recognized for their contribution to the tight regulation of gene activation and silencing. Hence, it is not surprising that deregulation of one of the epigenetic mechanisms and the transcription machinery might lead to disruption of memory associated gene expression, resulting in a number of syndromes associated with mental disorders.

In the next sections I will discuss about chromatin and transcription factors and their importance in the gene expression.

1.1 Chromatin modifications and the regulation of gene expression

The human genome consists of over three billion base pairs (bp), which If stretched out, would form very thin thread, about 2 meters long(Alberts, 2004). In order to fit the long strands of DNA into the nucleus, which is only 10 μm in diameter, nature requires a sophisticated way to pack the DNA without compromising its accessibility (Fig 1.1). DNA is wrapped around an octamer complex of histones to form nucleosomal units, giving the appearance of beads on a string (Lander et al., 2001). The histones are small proteins with a positive charge, which enable them to interact with the negative backbone of DNA. Each nucleosomal unit consists of about 200 bp of DNA, of which 147 bp are wrapped around the histone octamer complex and the remaining base pairs form a linker to the next nucleosome (linker DNA). Each histone octamer complex contains two copies of histones H3 and H4 bordered by two copies of histones H2A and H2B(Kornberg and Lorch, 1999).

Each histone has a long N-terminal 'tail' extension that protrudes from the nucleosome core (Fig 1.2). These tails are the main target for histone modification and serves as a binding site for gene activators and repressors. The histone tails are also thought to create a higher level of chromatin folding by interacting with other nucleosomes(Dorigo et al., 2004; Kornberg and Lorch, 1999). The nucleosome structure is further compacted into a solenoid structure in genes that are not transcriptionally active or about to become transcriptionally active. On the

other hand, active or potentially active genes are found in an open nucleosomal structure.

Figure 1.1 Organization of DNA in the eukaryotic nucleus

The figure was obtained from www.Epitron.eu

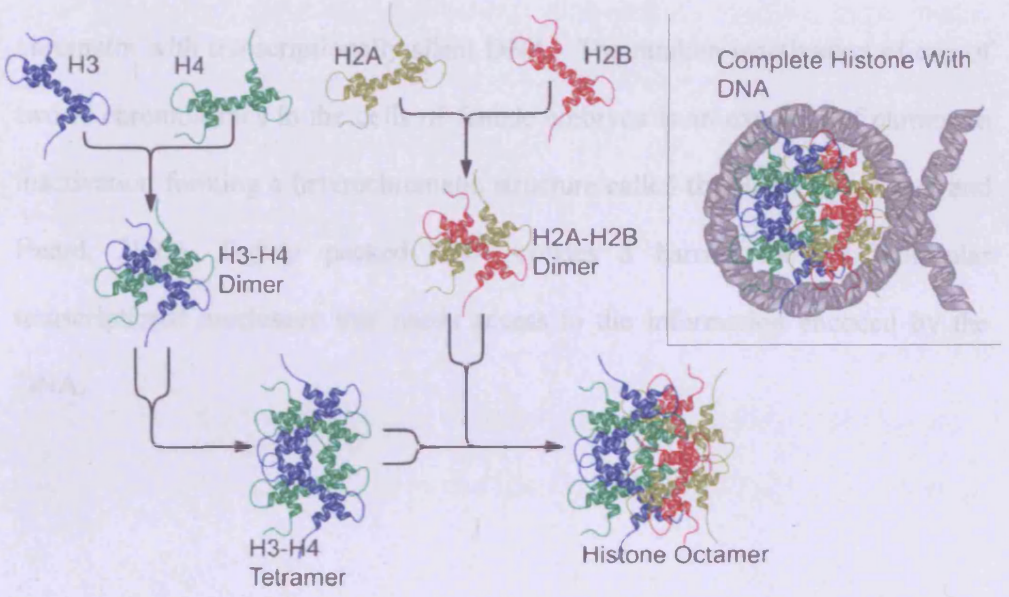

Figure 1.2 The assembly of the core histones into the nucleosome

Each of the core histones contains a histone-fold domain that allow heterodimeric interactions between core histones. The H3-H4 tetramer forms the scaffold of the octamer onto which two H2A-H2B dimers are added to complete the assembly.

Chromatin can be found in two different states; euchromatin and heterochromatin. Euchromatin is generally regarded as an open chromatin structure associated with active genes, whereas heterochromatin is considered to be tightly packed chromatin with transcriptionally silent DNA. The random inactivation of one of two X chromosomes in the cells of female embryos is an example of chromatin inactivation forming a heterochromatic structure called the Barr body(Avner and Heard, 2001). Tightly packed DNA creates a barrier for the molecular transcriptional machinery that needs access to the information encoded by the DNA.

1.2 Biochemistry of chromatin

Biochemical activities that modify chromatin structure are usually divided into three groups (i) chromatin remodelling catalyzed by specific large protein complexes containing specific enzymes that hydrolyze ATP (ii) covalent posttranslational modification of histone tails (iii) histone variants with specialized properties.

Since one of the main topics of this thesis is histone post-translational modification, the role of chromatin remodelling by ATP hydrolysis protein or the role of histone variants will not be discussed in this thesis (For review (Li et al., 2007).

1.2.1 Histone modifications

The histone cores and in particular their tails, are targets for a considerable number of covalent modifications, such as acetylation, ubiquitination, phosphorylation and methylation(Biel et al., 2005). Conventionally, some histone modifications are associated with active gene activation, such as H3 Lys4 methylation and H3 Lys56 acetylation. On the other hand, other histone modifications associated with the inactivation of gene transcription have been identified, such as H3 Lys27 methylation and H2A Lys119 ubiquitination. Histone modifications both individually and in combination have been proposed to be involved in gene regulation by modifying the chromatin accessibility and by

acting as docking sites for transcription factors (see section XXX)(de la Cruz et al., 2005; Strahl and Allis, 2000). Interestingly, different histone modifications can interact with one another. For example, H3 Lys9 demethylation facilitates H3 Ser 10 phosphorylation and Lys14 acetylation. Post-translational modifications in nucleosomal histones and their combinations seem to be related with, and even predict, transcriptional states. This led to the idea of a histone code whereby "distinct histone amino-terminal modifications can generate synergistic or antagonistic interaction affinities for chromatin-associated proteins"(Jenuwein and Allis, 2001). As this thesis is mainly concerned with histone acetylation, other modifications will be discussed only briefly.

(a) Histone methylation

Histone methylation, was first described in 1964(Murray, 1964), and histone methyltransferase enzymes (HMT) were found to act on the lysine or arginine residues of the histone tail(Peterson and Laniel, 2004). For a long time methylated histone was thought to be highly stable and only recently a histone demethylase was discovered(Shi et al., 2004). The methylation of histones plays an important role in chromatin regulation and is linked to both gene expression and silencing(Zhang and Reinberg, 2001). Methylation of histone H3 on Lys9 is associated with heterochromatin formation and gene repression(Nakayama et al., 2001). In contrast, methylation of H3-K4 is linked to gene activation(Santos-Rosa et al., 2002).

(b) Histone phosphorylation

Phosphorylation can occur at serines and threonines of all the histone tails. Histone phosphorylation was found to be involved in DNA repair, apoptosis and mitotic chromosome condensation(Peterson and Laniel, 2004). Phosphorylation of H3 on its Ser10 is involved in gene activation by promoting H3 Lys14 acetylation(Agalioti et al., 2002). Moreover, initial studies showed that histone phosphorylation has a role in transcriptional induction of immediate early genes in mammalian cells, such as the c-Fos gene(Mahadevan et al., 1991). Finally, heat-shock gene induction in Drosophila is accompanied by dramatic increases in histone H3 Ser-10 phosphorylation that occurs at transcriptionally active loci(Nowak and Corces, 2000).

(c) Histone acetylation

One of the most studied histone modifications is the acetylation of the evolutionary conserved lysine residues on the histone N-termini by histone acetyltransferases (HAT). Lysine acetylation was found to change the net charge of nucleosomes, resulting in the relaxation of DNA-histone interactions, altering the chromatin structure that enables gene transcription. Moreover, histone acetylation was shown to enhance binding of several gene activation factors, which contain a region known as the bromodomain. Thus, the bromodomain binds with greater affinity to histones when specific lysines are acetylated. In contrast, histone deacetylation, catalyzed by histone deacetylases (HDAC), was found to package the DNA into a more condensed form, limiting the access of transcription factors and thus acting as a gene silencer(Ng and Bird, 2000). Both HATs and HDACs (Fig 1.3) generally lack intrinsic DNA-binding activity. Instead, they are

recruited to DNA by sequence-specific transcription factors to stimulate either transcriptional activation or repression (see section 1.4). Interestingly, Levenson *et al.* (2004) were able to show that increased acetylation of histone 3 in area CA1 of the hippocampus occurs following contextual fear conditioning, a form of associative memory in which mice associate a novel context with an aversive stimulus. Additionally, by inhibiting HDAC, they were able to manipulate changes in the chromatin that might enhance transcription of genes associated with the formation of LTM(Levenson et al., 2004).

Figure 1.3 Histone acetylation and deacetylation

Transcriptional activators and repressors are associated with co-activators and co-repressors, which have HAT and HDAC activities, respectively. Histone acetylation is characteristic of actively transcribed chromatin and may weaken the binding of histones to DNA or alter their interactions with other proteins (modified from (Cooper, 2000)).

1.3 HATs and HDACs

1.3.1 HDACs

Mammalian HDACs can be divided into the classical and the silent information regulator 2 (Sir2)- related protein (sirtruin) families(Yang and Seto, 2007). In humans, members of the classical family have another subdivision, which include class I, II and IV, that share sequence similarity and require Zn^+ for deacetylase activity. Class I HDACs (HDAC1-3, HDAC8) are related to the yeast gene repressor Rpd3p, and are subunits of at least two distinct co-repressor complexes, the Sin3 complex and the NuRD complex. Class II HDACs (HDAC4-7, 9 and 10) are similar to the yeast Hda1p HDAC, they act as gene repressors and have been implicated in various roles in cell differentiation and development. Class IV comprises HDAC11, which has some features of both class I and II HDACs. The sirtruin family includes class III HDACs (SIRT1-7), which are similar to yeast Sir2. Class III HDACs are biochemically and structurally distinct from the classical family and require NAD^+ as a cofactor. HDACs appear to be involved in gene silencing and heterochromatin formation at centromeres and telomeres (for review (Gregoretti et al., 2004)).

1.3.2 HATs

HATs were found to be involved in histone acetylation (leading to gene activation), chromosome decondensation, DNA repair and non-histone substrate modification. All HATs share a highly conserved motif containing an acetyl-CoA binding site (Roth et al. 2001) and can be subdivided into several classes based on their sequence homologies. Historically, HATs have been divided into type A

(nuclear), and type B (cytoplasmic) HATs. However, this distinction between B and A type of HATs is somewhat unclear, since HATs can shuttle between nucleus and cytoplasm. More accurately, HAT complexes can be classified into several subfamilies based on their sequence homologies (Table 1.1).

The general control non-derepressible 5 (Gcn5) is the founding member of the Gcn5 N-acetyltransferases (GNATs). The GNAT family members include Gcn5, PCAF, Elp3, HAT1m Hpa2 and Nut1. The MYST family is named after the founding members of the family: Morf, Ybf2, Sas2 and Tip60(Kimura et al., 2005).In addition, other proteins including CBP/p300, Taf1 and a number of nuclear receptor co-activators have been shown to possess intrinsic HAT activity. However, these proteins do not contain a consensus domain and therefore represent an 'orphan class' of HAT enzymes(Kimura et al., 2005).

One characteristic of many, if not all, HATs is that they do not work in isolation *in vivo*. Interestingly, some HATs appear to associate with other HATs and co-activators for regulation of specific target genes. It was recognized that recombinant forms of several HATs, notably Gcn5, fail to acetylate nucleosomal substrates efficiently relative to mixes of free histone. Results with several HATs suggest that tail accessibility is a major factor affecting the rate of acetylation in oligonucleosome arrays(Herrera et al., 2000; Verreault et al., 1998), demonstrating that native HAT complexes, working alone or together with yet other chromatin remodeling complexes, such as SWI/SNF(Krebs et al., 2000), must in some way overcome the difficulty of tail accessibility in unmodified chromatin. To date, the best-characterized HAT complexes are those that contain

yeast Gcn5, such as SAGA and ADA(Grant et al., 1997; Sendra et al., 2000). Moreover, some HAT subunits were shown to have domains that cooperate to recruit the HAT to the appropriate location on the genome. These domains include bromodomains, chromodomains, WD40 repeats, Tudor domains and PHD fingers that bind to chromatin and recognise histone tails(Lee and Workman, 2007).

Group	HAT	Organism	Possible function	Ref
GNATs family	Gen5	yeast	transcriptional activation	(Brownell et al., 1996)
	Gen5L	mammal/fly	transcriptional activation	(Smith et al., 1998a; Xu et al., 1998)
	PCAF	mammal	transcriptional activation	(Yang et al., 1996)
MYST family	Tip60	mammal	transcriptional activation/DNA repair	(Yamamoto and Horikoshi, 1997)
	HB01	mammal	gene expression?/DNA replication?	(Iizuka and Stillman, 1999)
	MORF	mammal	transcriptional activation	(Champagne et al., 1999)
	MOZ	mammal	transcriptional activation	(Champagne et al., 2001)
	MOF	mammal/fly	transcriptional activation	(Hilfiker et al., 1997)
	Esa1	yeast	transcriptional activation	(Smith et al., 1998b)
	Sas3	yeast	transcriptional activation?	(Takechi and Nakayama, 1999)
	Sas2	yeast	anti-silencing	(Kimura et al., 2002; Sutton et al., 2003)
'Orphan' class	HAT1	yeast	histone deposition	(Kleff et al., 1995)
	p300/CBP	mammal	transcriptional activation	(Ogryzko et al., 1996)
	$TAF_{II}250$ (TAF1)	mammal/fly/yeast	RNA pol II transcription	(Mizzen et al., 1996)
	ACTR/SRC-1	mammal	transcriptional activation	(Chen et al., 1997; Spencer et al., 1997)
	Elp3	yeast	transcriptional elongation	(Wittschieben et al., 1999)
	hTFIIIC110	mammal	RNA pol II transcription	(Kundu et al., 1999)
	hTFIIC90	mammal	RNA pol II transcription	(Hsieh et al., 1999)2
	Hpa2	yeast	?	(Angus-Hill et al., 1999)
	Nut1	yeast	RNA pol II transcription	(Lorch et al., 2000)
	ATF-2	mammal	transcriptional activation	(Kawasaki et al., 2000)

Table 1.1 A list of identified histone acetyltransferase.

1.4 Transcription

The tightly regulated expression of the thousands of genes of the eukaryotic genome is crucial for the proper development and the normal functioning of all organisms. Once the chromatin has been opened it then needs to be transcribed. Different genes are transcribed in different cell types leading to the production of their corresponding proteins. In addition, different stimuli will trigger new protein synthesis by activating transcription of specific genes. Control over gene expression is achieved by regulation of transcription, the process by which DNA is copied into RNA transcript. In a eukaryotic cell, the transcription process requires three members of the RNA polymerase family. RNA polymerase I and III, which transcribe respectively rRNA or tRNA and other small RNAs, and RNA polymerase II, which transcribes the vast majority of protein coding genes. Although prokaryotic RNA polymerase can initiate transcription on its own, eukaryotic polymerases require the prior assembly of transcription factors at short defined segments of double-helical DNA (gene promoter).

Transcription factors, which utilize RNA polymerase II, are divided into two main groups(Villard, 2004):

1. Basal transcription factors, which recruit the RNA polymerase II multi-protein complex to a promoter, which often contains a conserved DNA sequence motif called the TATA box (Fig 1.4).
2. Gene-specific transcription factors, which bind to a specific DNA sequence and regulate the basal transcription complex either by activating or repressing it.

Generally, regulatory transcription factors bind to gene promoters and activate or inhibit transcription. Numerous transcription factors have been identified in a wide variety of organisms. Many of these transcription factors are activators and have several modules in common: (1) A DNA-binding domain that positions the protein on specific sequences. (2) Activation domains that are thought to work by recruiting or accelerating the assembly of the general transcription factors on the promoters. Transcription factors that do not bind to the DNA directly but link a DNA-binding transcriptional activator to the basal transcription complex are referred to as co-activators. On the other hand, a number of cases have now been demonstrated in which a transcription factor exerts an inhibitory effect, directly or indirectly, on transcription initiation(Latchman, 2007). Since this thesis deals mainly with transcription activation, transcription repression will not be discussed (For review(Latchman, 2007)).

Figure 1.4 RNA Polymerase II Transcription-Initiation Complex

Stepwise assembly of a transcription-initiation complex from isolated RNA polymerase II and basal transcription factors (figure modified from (Lodish H., 2000)).

1.4.1 DNA binding domains

Research into the DNA binding domains of different transcription factors have shown that there are several groups with common structural elements that are able to bind to specific DNA sites. The ability of transcription factors to bind to DNA is of great importance since DNA-binding serves as the first step of the transcription process. A DNA binding domain can recognise DNA via a variety of specific structural motifs, such as helix-turn-helix motif, zinc finger motif and the basic DNA binding domain. The basic DNA-binding domain, which is associated with a leucine zipper motif or a helix-loop-helix motif, possesses the unique ability to bind DNA as a dimer of two transcription factors. In addition, the basic DNA-binding domain is characterised by a large positive charge, which allows the binding domain to be structured into an α-helix when interacting with DNA(Weiss et al., 1990). The exact amino acid sequence that is folded into the DNA-binding motif determines the particular DNA sequence that is recognised(Alberts, 2004).

1.4.2 Activation domains

Current evidence indicates that following DNA binding, a transcription factor influences gene expression through its trans-activation domain. In most cases the activation domain ranges from 30 to100 amino acids in length and falls into three distinct families having respectively; acidic, glutamine-rich and proline-rich domains(Latchman, 2007). Activation domains may act directly, or may recruit a co-activator that possesses activation properties and the ability to interact with the basal transcription complex. It seems that binding of the basal transcriptional complex to the TATA box produces only a low rate of transcription; however, this

rate can be greatly increased by the binding of other transcription factors to upstream sequences. Such interactions seem to enhance transcription by increasing the rate of transcription factor complex assembly or by stimulating the level of its activity(Latchman, 2007).

1.5 The regulation of transcription factors

As mentioned above, transcription factors play a key role in the transcription of genes. Regulation of gene expression can be achieved through control of transcription factors either by regulating their synthesis or activating their inactive form by post-translational modification(Latchman, 2007).

1.5.1 Regulation of transcription factor synthesis

Specific transcription factors are synthesised at certain stages of development and in specific cell types, thus resulting in tissue-specific gene expression. One such example is the Myod transcription factor. Synthesised by skeletal muscle cells, Myod was found to regulate gene expression of for example, creatine kinase. Interestingly, it was shown that artificial induction of Myod expression in other cell types, such as fibroblast cells, was sufficient to convert them into skeletal muscle cells (Weintraub et al., 1989).

1.5.2 Regulation of transcription factor activity

Synthesis of transcription factors is an important control point in transcription factor regulation, however it is not sufficient. It is also necessary to have a mechanism by which pre-existing transcription factors can be transformed from an inactive to an active form by posttranslational changes. Such activation can take place through several mechanisms, such as ligand binding, protein-protein interaction and protein modification (Fig 1.5).

An example of a transcription factor whose activity is regulated is CREB (cyclic AMP response element (CRE) binding protein). CREB, which has a basic DNA

binding domain, was shown to bind to the palindromic CRE sequence, TGACGTCA, as a dimer through each CREB monomer binding to one half of the CRE sequence. The CREB factor is found in cells in its inactive form prior to its activation following cyclic AMP treatment. Moreover, CREB can be found bound to CRE before exposure to cyclic AMP but the DNA-bound CREB does not activate transcription. An increase in levels of cyclic AMP leads to the activation of protein kinase A (PKA) that rapidly phosphorylates CREB at Ser133(Gonzalez and Montminy, 1989). The Ser133 is located in an area of CREB that is referred to as a the phosphorylation box, which is flanked by glutamine-rich amino acids that act as a transcriptional activation domain(Latchman, 2007). Upon phosphorylation of Ser133, CREB undergoes a structural change that allows it to activate transcription (Fig 1.6). CREB Ser133 phosphorylation is considered to be the key event that mediates the initiation of transcription since mutation of Ser133 to alanine abolishes transcription(Gonzalez and Montminy, 1989).

Figure 1.5 Transcription factor activation mechanisms

Mechanisms of transcription factor activation from an inactive to an active form by posttranslational changes. a) Ligand-mediated conformational change b) Removal of an inhibitory protein c) Protein modification d) Transcription factor stabilization in order not to get degraded (modified from (Latchman, 2007)).

Figure 1.6 CREB activation

The ability of DNA-bound CREB to activate transcription is produced by the cyclic-AMP dependent activation of PKA, which phosphorylates the CREB protein resulting in its activation (modified from (Latchman, 2007)).

1.6 CBP and p300 transcriptional activity

As mentioned in section 1.5.2 the phosphorylation of CREB on Ser 133 activates transcription. To identify the mechanism of this effect, Chrivia *et al.* screened a cDNA expression library with Ser 133 phosphorylated CREB in order to identify proteins that would interact with phosphorylated CREB. This screen resulted in the isolation of cDNA clones encoding CREB binding protein (CBP)(Chrivia et al., 1993). CBP is a 265 kDa co-transcription factor that binds to CREB only in its phosphorylated form (Fig 1.7) (Goodman and Smolik, 2000). CBP binding only to phosphorylated CREB suggested that CBP plays a role in the ability of CREB to activate transcription. In agreement with this, treating cells with antibodies to CBP prevented gene transcription in response to cAMP, indicating the importance of CBP for this effect(Giordano and Avantaggiati, 1999).

CBP displays great homology to another nuclear protein, p300, which was isolated independently from CBP (Eckner et al., 1994). Despite their high similarity no link was made between CBP and p300, until 1994. It is now known that CBP and p300 have similar functions as extremely versatile transcriptional co-activators(Arany et al., 1994). CBP and p300 (CBP/p300) were found to play a double role in gene transcription: 1) CBP/p300 link between DNA-binding transcription factors and the basal transcription machinery, providing a scaffold for transcription factor interaction 2) CBP/p300 modify histones by acetylation of specific lysine residues. (Fig 1.7)(Victor et al., 2002). Thus CBP/p300 were shown to bind to several components of the basal transcription machinery and CBP/p300 have been identified as part of the RNA polymerase II protein complex(Neish et al., 1998). Moreover, CBP/p300 were shown to have a HAT

domain and were shown to acetylate histone, which is associated with modifications of chromatin structure and an increase in gene transcription (section 1.2.1). Given the high structural and functional homology of CBP and p300, for the purposes of this thesis these proteins will be referred to as CBP/p300. Nevertheless, when reviewing studies conducted on only one member of the family, reference is made only to the family member under study.

Figure 1.7 CBP association with the basal transcription machinery

CBP/p300 are recruited to act as bridge between DNA-bound phosphorylated CREB and the basal transcription machinery located at the start site of transcription. In addition CBP/p300 act as a histone acetyltransferase, making the chromatin more accessible (figure was modified from (Lodish H., 2000)).

1.6.1 CBP/p300 interactions and functions

Although CBP was originally identified as a co-activator for CREB, it was later shown that CBP or p300 are important co-activators for a variety of factors, such as p53, MyoD, AP1, nuclear receptors and many other proteins (Fig 1.8). It was also shown that CBP/p300 bind with TBP, TFIIB, TFIID and RNA polymerase II, demonstrating their role in recruiting RNA polymerase II holoenzyme (Dallas et al., 1997; Kee et al., 1996). In addition, both CBP/p300 were shown to play a critical role in cardiac hypertrophy (Gusterson et al., 2003) and were demonstrated to be essential for modulating processes such as G1-S phase transition in the cell cycle(Ait-Si-Ali et al., 2000).

The interaction of CBP/p300 with an array of transcription factors that are involved in different aspects of gene regulation, such as STAT, NFkB and AP1, places CBP/p300 at the cross roads of a range of signalling pathways in the cell. Thus, this suggests that CBP/p300 have a vital role in a variety of cellular functions (Fig 1.9)(Goodman and Smolik, 2000). The relatively low abundance of CBP/p300 in the cell means that different signalling pathways compete for CBP/p300, which results in mutual antagonism between different competing pathways. An example of this is the growth promoting effect of the AP1 pathway compared to growth arresting effect of the p53 pathway(Avantaggiati et al., 1997; Kamei et al., 1996).

Figure 1.8 CBP/p300 organisation and interactions

Interacting proteins are shown at the top of the figure; functional domains are depicted below. Many known interactions are not included due to space limitations (figure was modified from Goodman 2000(Goodman and Smolik, 2000)).

Figure 1.9 CBP/p300 interactions with transcription factors and the signalling pathways that activate them.

(modified from (Latchman, 2007)).

1.6.2 CBP/p300 domain structure

In recent years several important amino acid domains have been identified in CBP/p300, as shown in Fig 1.8. These domains were found to be involved in transcription, activation or repression and protein-protein interaction. The N-terminal of CBP/p300 contains an LXXLL sequence motif, in which L represents leucine and X represents any amino acid. LXXLL. The LXXLL motif has been found to be necessary and sufficient for ligand-dependent interactions with the nuclear receptor ligand binding domain(Heery et al., 1997). In addition, the 586-665 aa region of CBP/p300, referred to as KIX, was found to bind to phosphorylated CREB. KIX binds to the kinase inducible domain of CREB and other transcription regulating proteins(Radhakrishnan et al., 1997). However, not all CBP domains are associated with gene activation. The 999-1057 aa region was shown to repress CBP activity through modification by SUMO-1(Kuo et al., 2005).

C-terminally from this domain, a bromodomain (1104-1176 aa) and a HAT domain have been described. The short bromodomain region binds acetylated lysine residues whereas the HAT domain, which occupies a central portion of CBP/p300, is involved in histone and non histone protein acetylation(Imhof et al., 1997). Importantly, a growing number of molecules, other than histones, were shown to be acetylated by CBP/p300. An important example of non-histone acetylation is the acetylation of p53 by p300, which increases the DNA-binding activity of p53(Gu and Roeder, 1997).

Moreover, CBP/p300 have three zinc finger motif domains, the first domain is located between 346-432 aa and binds several interacting factors. The second and third zinc finger regions lie in the HAT domain of CBP/p300 and were found to be necessary for CBP, but not p300, HAT activity(Bordoli et al., 2001a). Importantly, despite their structural role, none of these zinc finger motif domains make CBP/p300 a DNA binding protein. Lastly, both the N- and C-terminus of CBP/p300 were shown to contain an activation domain which were shown to increases the assembly of the transcriptional machinery (Kalkhoven, 2004).

1.6.3 CBP/p300 post-translational modifications

Accumulated evidence demonstrated that cellular signals can affect CBP directly and thereby modify transcriptional responses without affecting CREB phosphorylation. Numerous kinases have been suggested to be involved in such processes. The cell cycle-regulated cyclin E-Cdk2 complex, for example, was demonstrated to phosphorylate CBP *in vitro*(Banerjee et al., 1994) and thereby increase CBP HAT activity(Ait-Si-Ali et al., 1998). Moreover, several papers suggested that CBP is regulated by PKA, which was shown to phosphorylate CBP and increase CBP-dependent transcriptional response(Chrivia et al., 1993). Calcium-dependent pathways can equally trigger CBP phosphorylation, since CaMKIV was shown to phosphorylate CBP and increase its activity (Chawla et al., 1998). Furthermore, the MAPK family was also demonstrated to phosphorylate CBP(Janknecht and Nordheim, 1996), which was shown to increase CBP HAT activity(Ait-Si-Ali et al., 1998). In PC12 cells, NGF increases the transcriptional activity of CBP in a p42/p44 MAPKs-dependent manner (Liu et al., 1998). Subsequent studies reported that p44MAPK could phosphorylate CBP *in vitro* and suggested that this modification increased CBP HAT activity(Ait-Si-Ali et al., 1999). Additionally, CCAAT/enhancer-binding protein (C/EBP) family members were shown to recruit CBP and trigger its phosphorylation(Kovacs et al., 2003). It was therefore proposed that CBP phosphorylation might induce HAT function by changing the intermolecular conformation of CBP.

On the other hand, phosphorylation at serine 89 residue of p300 by PKC at was demonstrated to reduce p300-dependent transactivation and that this reduction is

probably as a result of a reduction of the interaction of p300 with specific transcription factors(Yuan and Gambee, 2000). However, PKC phosphorylation at the serine 436 residue of CBP was found to enhance the ability of CBP to be recruited by AP-1 and Pit-1 transcription factors, and thus increase gene transcription(Zanger et al., 2001). Thus, this serine 436-residue phosphorylation of CBP allows it to preferentially active different pathways under different conditions.

Moreover, CBP/p300 are modified by methylation on specific arginine residues that is essential for stabilizing the structure of the KIX domain. This CBP/p300 methylation was found to affect CBP/p300 ability to bind to some of the transcription factors with which they interact. Thus, CBP/p300 methylation abolishes their ability to bind to the CREB factor but has no effect on the ability of CBP/p300 to bind to nuclear receptors(Xu et al., 2001). Hence, the competition between different transcription factors for binding CBP/p300 can be altered by modification of the co-activator.

1.6.4 CBP/p300 in embryogenesis

Homozygous $p300^{-/-}$ knock-out mice die early in embryogenesis, with lethality occurring between embryonic day 9 and 11.5. Several different factors seem to be involved in the lethality of the knockout mice as they display defects in cell proliferation, heart development and closure of the neural tube. Due to the early death of $p300^{-/-}$ mice, it is not possible to observe the influence of p300 deficiency on later events in embryogenesis, which may also be critically dependent on p300 function(Yao et al., 1998). Moreover, heterozygous $p300^{+/-}$ mice also die *in utero*,

suggesting that the normal dosage of p300 is limiting. In addition, while p300 heterozygotes have an increased incidence of lethality, CBP heterozygotes are viable(Partanen et al., 1999; Tanaka et al., 1997).

Mice with homozygous CBP mutations in mice are not viable and they display similar defects to mice lacking p300(Kung et al., 2000; Oike et al., 1999b; Yao et al., 1998). However, while $p300^{-/-}$ knockout mice exhibit abnormal heart formation, $CBP^{-/-}$ mice show normal heart formation. Additionally, the $CBP^{+/-}$ /$p300^{+/-}$ double heterozygote mice are also embryonic lethal, which supports the idea that critical events in development are sensitive to the overall CBP and p300 gene dosage. This data suggests that CBP and p300 exert certain common embryonic survival functions and that the combined dose of CBP and p300 is critical for mouse embryonic development. On the other hand, overexpression of CBP in *Drosophila* embryos was found to be lethal demonstrating the tight regulation of CBP/p300 (Marek et al., 2000).

1.7 CBP and p300 and their roles in diseases

1.7.1 CBP and Rubinstein-Taybi Syndrome

Rubinstein-Taybi syndrome (RTS) is a genetic disease characterised by mental retardation, broad thumbs and toes, short stature, distinctive facial features, skeletal abnormalities and increased risk of cancer(Rubinstein and Taybi, 1963). The incidence of RTS is estimated at 1/125,000 births and accounts for 1/300 cases of institutionalized mentally retarded people. Originally, the disease was found to be caused by mutations in CBP (Petrij et al., 1995). CBP has been found to be involved with 50% of RTS cases (Bartsch et al., 2005) and a very small percentage carry mutations in p300 (Roelfsema et al., 2005). The types of CBP mutations associated with RTS include missense mutations, translocations, inversions and microdeletions.

Wide variation exists in the severity of the mental deficiency and other clinical manifestations of RTS. The intelligence level of different RTS patients varies between the low IQ scores of 25 to 79. Other variable findings are congenital heart defects, renal abnormalities and failure of the testes to descend into the scrotum (cryptorchidism).

The variable clinical manifestations seen in RTS could be due to two different mechanisms of CBP dysfunction. The first mechanism of CBP dysfunction is haploinsufficiency, where there is only one functional copy of the CBP gene, which is not sufficient to produce enough protein to reach a wild-type condition. The second mechanism is a dominant-negative mutation of CBP, where abnormal

product derived from the mutant allele inhibits the wild-type product. To date, evidence supporting both mechanisms has been reported(Alarcon et al., 2004; Oike et al., 1999a). Thus, it seems likely that both mechanisms of CBP dysfunction can play a role in the development of RTS phenotypes.

In mice, a heterozygous deletion leads to a phenotype similar to RTS, which includes deficits in various forms of long-term memory(Alarcon et al., 2004; Oike et al., 1999b). Importantly, in RTS cases where CBP is truncated it was shown that CBP lacks part of its HAT region, thus impairing its ability to acetylate histones(Murata et al., 2001). Additionally, some CBP missense mutations were also shown to annul CBP HAT activity, suggesting that two sets of functional CBP HAT are needed to prevent RTS. Moreover, mutations that are found outside of the HAT domain might also contribute to diminished HAT activity. This is due to the regulation of the HAT domain of CBP by different regions in CBP, such its bromodomain.

The data above demonstrate that CBP is highly involved in RTS. It is hard to identify if RTS phenotypes arise from either CBP dysfunction in the nervous system during development or CBP dysfunction in neurons during adulthood. To address this issue, Korzus *et al.* generated inducible dominant-negative CBP (CBP[HAT-]) mice(Korzus et al., 2004). The CBP[HAT-] line-inducible CBP has two amino acid substitutions, which eliminate CBP HAT activity. The CBP[HAT-] induced adult mice exhibited normal short-tem memory while LTM was impaired. Interestingly, Korzus *et al.* were able to rescue the impaired LTM by suppression of the transgene expression and by administration of a histone

deacetylase inhibitor, suggesting that a pharmacological manipulation of HAT activity might provide a possible therapeutic method for treating RTS symptoms. Moreover, this might suggests that RTS LTM defects are as a result of CBP dysfunction in adult brain and not as a product of abnormal brain development.

1.7.2 Huntington's disease

Huntington's disease (HD) is an autosomal dominant neurological disease characterized by movement dysfunction and cognitive abnormalities. HD, which affects 1/10,000 people, is a dominant genetic defect is caused by expansion of an unstable CAG repeat in the huntingtin gene (Htt) resulting in a greatly expanded polyglutamine (polyQ) tract in the protein it encodes(Gusella and Macdonald, 2007). It is seems that in HD patients that tracts of 37 or more CAGs cross a pathogenetic threshold while normal individuals have 7-34 CAG repeats(Gusella and MacDonald, 2000). Despite its size (348kDa), Htt is soluble and its wild-type form was found to play a role in embryogenesis(Cattaneo et al., 2001), vesicular transport(Trushina et al., 2004) and in addition it may possess neuroprotective activity(Zhang et al., 2006).

The well-characterized HD mice models (Mangiarini et al., 1996; Schilling et al., 1999; Yamamoto et al., 2000) have greatly enhanced our understanding of the disease, however the mechanism by which Htt elicits its toxic effect is unclear. Several mechanisms such as oxidative damage, mitochondrial dysfunction and transcriptional deregulation were proposed as pathogenic mechanisms(Browne and Beal, 2004).

One mechanism by which Htt mutants deregulate transcription is by interaction with CBP(Nucifora et al., 2001). Colocalization of CBP with polyQ aggregates has also been observed in cells in culture, transgenic mice, and postmortem HD brain tissue(McCampbell et al., 2000; Nucifora et al., 2001). Moreover, not only did the Htt mutant protein bind to CBP, it also sequesters CBP into protein aggregations (Fig 1.10)(McCampbell et al., 2000; Nucifora et al., 2001). In addition histone acetylation is reduced in the R6/2 mice, which might be a result of the loss of soluble levels of nuclear CBP(Ferrante et al., 2003). Since the amounts of CBP in the cell are limiting, this prevents its binding to transcriptional activators and therefore causes disease(Nucifora et al., 2001). Furthermore, experiments in Drosophila polyQ disease models demonstrated that the progression of the disease is inhibited by HDAC inhibitors and thus suggest that the CBP HAT domain is involved in the disease(Steffan et al., 2001).

Figure 1.10 Mechanisms leading to CBP loss of function

CBP loss of function has been observed in different contexts of neurological disorders. First, RTS outcomes from a mutation on an allele of the CBP gene that results in decreased amounts of functional CBP protein. Second, in several cases of polyQ diseases, decrease in the amount of available CBP can be achieved by sequestration of the protein by mutated polyQ proteins, forming aggregates in the cytoplasm or the nucleus (figure modified from (Rouaux et al., 2004)).

1.8 Alzheimer's disease

Alzheimer's disease (AD) is the most common form of dementia, affecting approximately 5% of the population over the age of 65 years in Europe(Lobo et al., 2000). Extensive literature suggests that AD begins as a synaptic disorder that progressively involves larger areas of the brain, resulting in deteriorating memory (Cullen et al., 1997; Itoh et al., 1999; Masliah, 1995; Vitolo et al., 2002; Walsh et al., 2002).

In this regard, β-amyloid (Aβ), a major component of the amyloid plaques that constitute a neuropathologic hallmark of the disease(Fig 1.11), has been found to inhibit Long-term potentiation (LTP), a physiologic correlate of memory (Cullen et al., 1997; Vitolo et al., 2002). The amyloid plaque is a heterogeneous class of protein aggregates with β-pleated structure. Amyloid plaques are varyingly composed of compact or non-compact amyloid, and can be associated with degeneration of nearby neuronal cell processes(Dickson, 1997). The amyloid deposits are composed of straight, unbranching fibrils of 6-10nm at the ultrastructural level. Seminal work by Glenner and Wong identified these deposits to be composed mostly of the aggregated 4 kDa Aβ protein(Glenner and Wong, 1984). Aβ is heterogeneous in size, but the predominantly Aβ is found as Aβ40-42(Iwatsubo et al., 1994). Moreover, control subjects with high plasma Aβ42 levels have an increased chance of developing AD(Mayeux et al., 2003) (Mayeux et al 1999). Aβ is the proteolysis product of a larger precursor protein, the amyloid precursor protein (APP), which in its mutant form has been found to cause the familial Alzheimer's disease (FAD)(Kang et al., 1987).

The next two AD associated genes discovered were presenilin (PS) 1 and PS2(Levy-Lahad et al., 1995; Sherrington et al., 1995) and these were found to be the primary cause of familial AD (FAD)(Czech et al., 2000). PS1 is a part of the γ-secretase complex which is responsible for the cleaving of APP, leading to the increased production of Aβ42 peptide(Takahashi et al., 1999) suggesting a toxic gain-of-function pathogenic mechanism.

Studies on human Aβ-producing transgenic (Tg) mice have often revealed significant deficits in hippocampal basal synaptic transmission (BST) and/or LTP (for a review see (Sant'Angelo et al., 2003)). Furthermore, recent studies indicate that the expression of FAD-linked mutant PS1 in neural and primary cell lines leads to sensitivity to apoptosis, implying that neurotoxicity by FAD genes may play a role in the pathogenesis of AD(Czech et al., 1998; Hashimoto et al., 2004; Weihl et al., 1999; Zhang et al., 1998).

Another hallmark, in addition to the amyloid plaques, of AD is the formation of neurofibrillary tangles (Fig 1.11)(NFTs). In the mid 1980s, a number of laboratories discovered that the main protein composing NFTs was the microtubule-associated protein, tau(Grundke-Iqbal et al., 1986; Kosik et al., 1986). In Alzheimer's, tau deforms and loses its ability to support the cell, eventually aggregating into NFTs. Moreover, NFTs occur in the regions of the brain responsible for the various cognitive domains that are compromised during the course of AD and the density of tau inclusions correlating well with regional and global aspects of cognitive decline(Ghoshal et al., 2002; Mitchell et al., 2002). However, since this thesis focuses mainly on the on PS1 and its association with CBP, the tau theory will not be discussed further here.

Figure 1.11 Alzheimer's amyloid plaques and neurofibrillary tangles

The formation of amyloid plaques and neurofibrillary tangles are thought to contribute to the degradation of the neurons in the brain and the subsequent symptoms of Alzheimer's disease (Fig was taken from Alzheimer's Health Assistance Foundation website www.ahaf.org).

1.8.1 Genetics and risk factors for AD

AD is genetically complex and heterogeneous disorder. The prevalence of AD doubles every five years after 65 up to 90 and then remains stable. The most important risk factor for the development of AD is increasing age(Qiu et al., 2005). Other risk factors include family history of dementia, hyper and hypotension, high cholesterol, head trauma, low physical activity, obesity, low education, anaemia and heart failure(Atti et al., 2006; Kivipelto et al., 2001; Kivipelto et al., 2005; Qiu et al., 2006). As mentioned above, mutations leading to predominantly early onset of FAD have been characterized in three genes: APP on chromosome 21, PS1 on chromosome 14 and PS2 on chromosome 1. These mutations are inherited in an autosomal dominant manner with nearly 100% penetrance(Selkoe, 2001). However, mutations in these three genes account for less than 5% of all AD cases. The rest of the 95% of AD patients are by and large sporadic late onset cases with no recognizable pattern of classical mendelian inheritance. Sporadic AD patients share similar clinical and neuropathological features as the autosomal dominant AD patients, but the underlying genetic mechanism appears to be more complex.

Interestingly, in Down syndrome (trisomy 21), the triplication leads to overexpression of genes located on chromosome 21, including the APP gene. This results in the development of neuropathological changes similar to AD at the age of 40 years. At an age as early as 20-30 years these individuals develop amyloid plaques and later in life also neurofibrillary tangles(Mann et al., 1986).

1.8.2 Neuropathology of AD

Clinical symptoms are observed only late in the course of the disease, and the steady worsening of symptoms reflects the gradual development of brain destruction. This begins in a few limbic areas of the cerebral cortex, and then spreads in a predictable, non-random manner across the hippocampus, the neocortex and a number of subcortical nuclei (Fig 1.12) (Bráak and Braak, 1995). The limbic system is involved in maintaining the functions of memory and in establishing emotional aspects of personality. Atrophy of these regions leads to dementia.

During normal aging, changes occur in the anatomy of the brain. These alterations include an overall reduction in brain volume and weight, which are due to neuronal cell loss(Anderton, 1997). AD is a pathological form of aging and there is significant loss of large cortical neurons in AD brains compared to normal brains(Terry et al., 1981). Mounting evidence suggests that AD begins with subtle alterations of hippocampal synaptic efficacy prior to neuronal degeneration(Selkoe, 2002). Accordingly, the earliest symptoms appear to correlate with dysfunction of the cholinergic and glutamatergic synapses(Selkoe, 2002). Definitive diagnosis of AD is possible only upon autopsy.

As mentioned above, the two specific histopathological hallmarks of AD are the extracellular amyloid plaque and the intracellular NFTs. However, plaques and tangles are regularly found in autopsied brains of cognitively normal elderly individuals, this probably indicates the long preclinical phase of AD rather than plaques and tangles being normal in aging.

Figure 1.12 Major structures of the limbic system

Figure was taken from Le Moyne College website: http://web.lemoyne.edu/

1.8.3 APP processing

A central feature of APP biology is its the proteolysis. Upon synthesis, the APP molecule is proteolytically processed by so-called secretases. Three primary proteolytic cleavage sites were characteristics in APP. Two proteolytic cleavage sites were found close to the membrane and one site within the transmembrane domain and are termed α, β and γ cleavage sites (Fig 1.12). All cleavages are catalyzed by separate enzymes complexes referred to as α-, β- and γ-secretases. The α and β cleavages are separate events and each release a large extracellular domain of the protein, differing in size by only 17 amino acids at the c-terminus. The rest of APP is tethered to the membrane and is referred to as the c-terminal fragment, C99 for the β and C83 for the α-generated product. The transmembrane cleavage event catalyzed by γ-secretase occurs subsequent to α or β cleavage at several sites in close proximity within the c-terminal fragment sequence. Thus, after β cleavage, C99 may be processed by γ-secretase, which require the presence of PS proteins(Herreman et al., 2000), to generate the predominant Aβ40 or Aβ42 fragments, as well as fragments spanning Aβ39 and Aβ43, together with the remaining C-terminal tail. Similarly, after α cleavage, C83 can be processed by γ-secretase to generate p3 fragment, with the same C-terminal fragments.

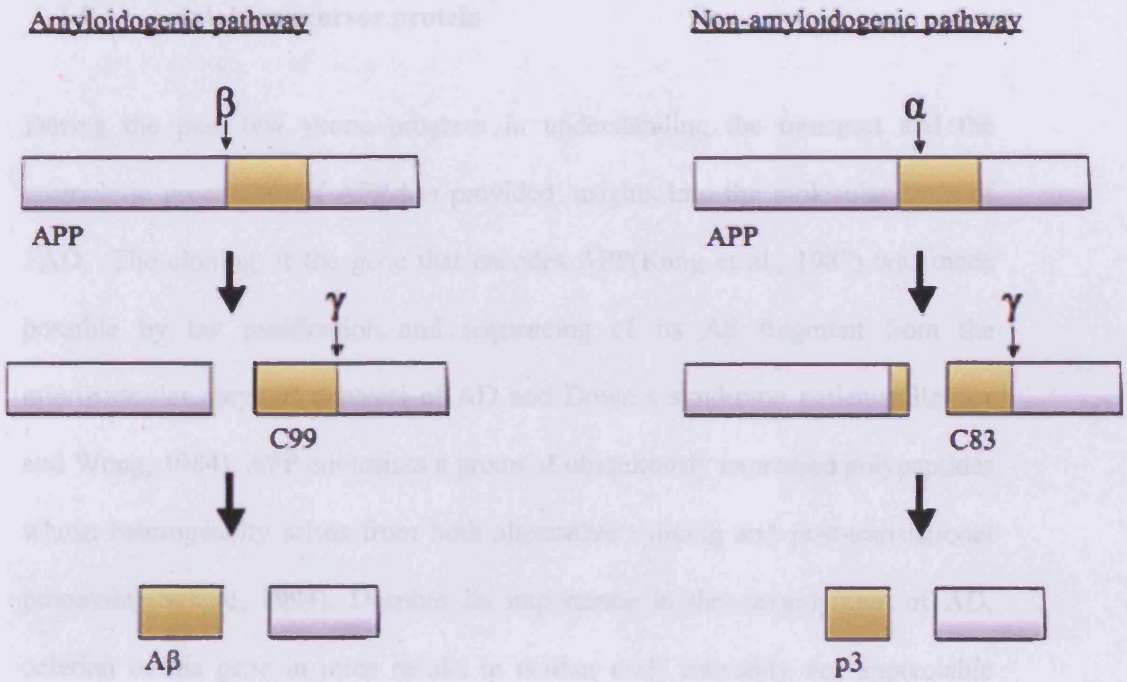

Figure 1.13 APP processing

In the left of the figure, APP is processed by β-secretase to generate C99. In the non-amyloidogenic pathway, α-secretase generates C83. The C-terminal fragments of both pathways are processed by γ-secretase generating Aβ or p3.

1.8.4 Amyloid precursor protein

During the past few years, progress in understanding the transport and the proteolytic processing of APP has provided insights into the molecular basis of FAD. The cloning of the gene that encodes APP(Kang et al., 1987) was made possible by the purification and sequencing of its Aβ fragment from the microvascular amyloid deposits of AD and Down's syndrome patients(Glenner and Wong, 1984). APP comprises a group of ubiquitously expressed polypeptides whose heterogeneity arises from both alternative splicing and post-translational processing(Selkoe, 1994). Despites its importance in the development of AD, deletion of the gene in mice results in neither early mortality nor appreciable morbidity.

The lack of a vital consequence of APP deletion in vivo may result from mammals expressing proteins that are closely homologous to APP, the amyloid precursor-like proteins (APLPs)(Wasco et al., 1992), but which do not contain the Aβ sequence. Thus, the prime function(s) of the molecule in vivo remain vague. However, functions that have been described in vitro include enhancement of cell-substrate adhesion, cholesterol metabolism and cognitive processes(Turner et al., 2003). However, no evidence has emerged that a basic cellular function of APP is lost in AD patients; instead, APP mutations seem to act by a gain of function mechanism, mostly the increased production of the cytotoxic Aβ fragment.

1.8.5 Presenilin 1

PS1 is expressed in most human tissues including embryonic and adult

brain(Kovacs et al., 1996; Lee et al., 1996). The highest concentrations in the brain are detected, according to in situ hybridization, within the neurons of the hippocampus, entorhinal cortex and low traces of transcript are also found in white matter glial cells. The hydrophobic PS1 protein consists of 467 amino acids and it is shown to span eight times the membranes(Hardy, 1997) (Fig. 1.14). The N- and C termini as well as the large hydrophilic loop between TM6 and TM7 are orientated towards the cytoplasmic side and are therefore available for interactions with various cytoplasmic proteins (Zhang et al., 1998). PS1 protein is mainly located within the ER, however, PS1 immunoreactivity has showed it can also be found in Golgi apparatus as well as plasma membranes and nuclear kinetochores.

Figure 1.14 PS1 molecular model

The eight putative transmembrane domains are depicted as open barrels 1-8, with the amino- and carboxy-terminal domains and large loop domain projecting into the cytoplasm. Residues associated with mutations found in familial Alzheimer's disease are coloured as indicated in the key. 'Endoproteolysis' indicates the approximate site of the imprecise cleavage of the molecule. (Figure was modified from(Tandon and Fraser, 2002)).

1.8.6 Functions of PS1

A major function of the PSs is their role within the γ-secretase complex, and in addition to APP, this complex processes a variety of other targets, for example Notch, LRP and Cadherin family members (Koo and Kopan, 2004). These additional targets are localised to detergent soluble membrane domains, indicating that these processing events are distinct from APP processing(Vetrivel et al., 2005). Moreover, several lines of evidence indicate that the functions of PS1 are related to apoptosis and the processing and trafficking of membrane proteins. In addition, the mutant of PS1 is known for its involvement in the APP processing, and its responsibility for the increased production and deposition of the amyloidogenic Aβ42(Citron et al., 1997; Scheuner et al., 1996). The following description of the functions of the PSs is by no means exhaustive, but provides an overview of perhaps the most relevant roles of this protein.

Notch processing

Studies conducted on the *Caenorhabditis elegans* homologue of PS1, SEL-12, and with PS1 knockout mice, has shown that PS1 may be involved in the processing and trafficking of membrane-bound proteins including the Notch receptor(Levitan and Greenwald, 1995; Wong et al., 1997). PS1 protein deficiency and the aspartate mutations in TM6 and TM7 of PS1 gene block the endoproteolytic cleavage of Notch. Notch intracellular cytoplasmic domain (NICD), which is released from the Notch after endoproteolysis, translocates to the nucleus, where it is needed for modifying transcription of specific genes, which regulate cell-fate during development(Schroeter et al., 1998).

Wnt signalling

The Wnt family are a group of extracellular glycoproteins that regulate homeostasis and development. Wnt signalling leads to the stabilisation of β-catenin and induction of gene expression(Nelson and Nusse, 2004). Thus, Wnt signalling increases cytosolic β-catenin, which translocates to the nucleus where it activates transcription of genes such as Cyclin D1.

WT PS1 interacts with components of the Wnt signalling cascade. It binds to β-catenin and negatively regulates both its stability and transcriptional activity(Killick et al., 2001). Moreover, both PS and β-catenin can be phosphorylated by p35/Cdk5(Lau et al., 2002; Munoz et al., 2007) and this may regulate their binding (Kesavapany et al 2001). On the other hand, PS1 mutations have reduced ability to bind to β-catenin, thus leading to increased β-catenin(Killick et al., 2001).

In addition, Wnt signalling may function in regulation of cognitive processes. Thus, the deregulation of endogenous β-catenin is observed in rats injected with preformed Aβ fibrils, and there is also evidence of neurodegeneration and behavioural impairments (De Ferrari et al., 2003).

1.9 CBP and Alzheimer's disease

It has been shown that wild type PS1 cleaves the transmembrane protein N-cadherin to release an intracellular C-terminal fragment. In turn, this fragment has been shown to bind to CBP and promote rapid proteasomal degradation, which downregulates CBP function (Marambaud et al., 2003). Moreover, it has been demonstrated that the mutant forms of PS1 found in FAD cannot cleave N-cadherin and therefore fail to generate the C-terminal fragment that will lead to CBP degradation. This suggests that the mutation in PS1 would result in enhanced levels of CBP and that CBP over-expression might be toxic.

It has also been shown that introduction of the FAD mutant PS-1/L286V caused dysregulation of TCF/β-catenin signalling, which has a central role in neuronal differentiation and proliferation. Thus, the PS-1/L286V mutant inhibits neuronal differentiation and the TCF/β-catenin/CBP interaction is involved in the dysregulation caused by the PS-1/L286V FAD mutant(Teo et al., 2005). However, it was shown that despite the homology high homology between p300 and CBP, the TCF/β-catenin/p300 interaction was not involved in the effect of PS-1/L286V mutant.

In contrast, previous investigations of normal presenilin function revealed a contradictory role of a PS1-CBP interaction in neurodegeneration. Thus, PS 1 and 2 conditional double knockout mice showed reductions in CBP levels and in CRE-dependent gene expression in the cerebral cortex, which is likely to contribute to subsequent neuronal degeneration(Saura et al., 2004). Interestingly, studies in cultured neurons show that Aβ42 inhibits activity-induced

phosphorylation of CREB, which serves as an essential step for CREB–CBP signaling(Gong et al., 2004). Moreover, it was shown that CBP loss takes place during neuronal death in models relevant to AD. During their experiments, Rouaux *et al.* demonstrated that when neurons were committed to die through the use of an antibody directed against the extracellular fragment of the APP, they displayed both CBP loss and histone deacetylation(Rouaux et al., 2003). Thus, reduced activity of the CREB–CBP pathway might offer a new possible AD pathogenic mechanism.

Saura *et al.* global reduction in CRE-dependent gene expression caused by loss of PS function is in direct disagreement with Marambaud *et al.*. In their report Marambaud *et al.* showed reduced N-cadherin processing in cultured PS1 null cells was found to cause decreased cytoplasmic retention of CBP and increased c-fos expression, which is a gene that is induced by CBP(Marambaud et al., 2003). In contrast, Saura *et al.* examined the expression of multiple CRE-dependent genes, including c-fos, by quantitative methods in the adult cerebral cortex, and identified a consistent reduction in CRE-dependent transcription in the absence of PS function. Moreover, Saura *et al.* identified a parallel reduction in both nuclear and cytoplasmic CBP levels as a consequence of PS inactivation(Saura et al., 2004). In view of this contradiction, it would be interesting to further investigate the potential role of CBP in Alzheimer's disease.

1.10 Aims and objectives

The closely related CBP and p300 co-activator molecules play a key role in memory and learning. To date, the role of CBP and p300 in AD, as it shown in the literature, is unclear and contradictory. The aim of this thesis was to investigate the role of CBP and p300 in AD in three main steps:

1. To look at the effect of WT and mutant PS1, a known mutation associated with AD, on the transcriptional activity of CBP and p300, and investigate the signalling pathways involved using chemical inhibitors. In addition, to examine the specific regions of CBP and p300 that are involved in changes of activity induced by WT PS1.

2. To use antisense CBP to investigate the effect of inhibiting CBP in the stimulus response to WT PS1 using c-fos, one of the genes induced in the brain by CBP, which is involved in memory formation, luciferase expression. Additionally, to study the involvement of CBP histone acetyltransferase activity in the stimulus response to WT PS1.

3. To determine whether inhibitors of histone de-acetylation re-establish normal synaptic function and memory in the Alzheimer's disease mouse model.

Chapter 2

2 MATERIALS AND METHODS

2.1 Laboratory reagents

2.1.1 General suppliers

General laboratory chemicals were of analytical grade and purchased from the following companies: Sigma-Aldrich Corporation, Poole Dorset, UK; VWR International Ltd., Lutterworth, Leicestershire, UK; Hoffmann-La Roche Ltd, Basel, Switzerland. Phosphate buffered saline (PBS) was made using PBS tablets (1 tablet in 500ml ddH20) from Invitrogen Ltd., Paisley, UK. All solutions were made with Millipore water and autoclaved where necessary. General laboratory plasticware was purchased from VWR International Ltd., Dorset, UK and Eppendorf UK Ltd., Cambridge, UK.

2.1.2 Bacterial reagents

Bacto®- Yeast extract, Bacto®- Tryptone and Bacto® Micro-agar for bacterial growth medium were from Duchefa Biochemie, Haarlem, Netherlands. XLI blue E.eali cells were purchased from Stratagene, La Jolla, California, USA.

2.1.3 Molecular reagents

Restriction and modifying enzymes and buffers were purchased from Promega, Southampton, UK. QIAprep Mini prep and Maxi DNA prep kits were from Qiagen Ltd, Crawley, UK. DNA sequencing was performed by MWG-Biotech AG, Ebserberg, Germany.

2.1.4 Western blotting reagents

Hybond™-c nitrocellulose membranes, protein molecular weight Rainbow™ marker, Enhanced Chemiluminescence system (ECL) and Kodak XOMAT imaging photographic film were purchased from Amersham Bioscience, Little Chalfont, Buckinghamshire, UK. 30% (w/v) acrylamidelbisacrylamide solution for polyacrylamide gels was obtained from Amresco Inc., Ohio, USA. Photographic developing and fixing chemicals were obtained from Xograph Healthcare Ltd., Tetbury, UK. Primary antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA and Cell Signaling Technology, Inc., Danvers, MA, USA. Secondary antibodies were purchased from DAKO Ltd., Glostrup, Denmark.

2.1.5 Tissue culture reagents

FuGENE 6 Transfection Reagent was obtained from F. Hoffmann-La Roche Ltd, Basel, Switzerland. Tissue culture reagents were purchased from Gibco, Paisley, UK. Disposable sterile 0.2 μm filters were purchased from Millipore, Watford, UK. All cell culture flasks were obtained from Nunc UK and plasticware from Falcon, UK.

2.1.6 Assay reagents

Luciferase reagent and lysis buffer were purchased from Promega, Southampton, UK. BCA Protein assay kit was from Pierce, Rockford, USA.

2.1.7 Equipment

Trans-Blot™ cell transfer tanks, Bio-Rad Laboratories Ltd., Hertfordshire, UK.

Syngene light box, Syntopics Ltd., Cambridge, UK.

Bio-Rad GS-800 densitometer, Bio-Rad Laboratories Ltd., Hertfordshire, UK.

TD-20/20 Luminometer, Turner Designs, Sunnyvale, California, USA.

Zeiss microscope, Carl Zeiss MicroImaging, Inc., New York, USA.

BioRad GS800 Densitometer, Bio-Rad laboratories Ltd., Hertfordshire, UK.

Labsystems Multiskan RC Plate reader, Finland.

Leica TCS SPE confocal microscopy, Leica Microsystems, Mannheim, Germanny

2.2 Cell culture

All cell culture work was carried out under sterile conditions in a laminar flow cabinet. Media and reagents were purchased sterile, autoclaved or filtered with O.2μm filters.

2.2.1 F11 cells

F11 cells (F11/EcR cells) stably overexpress both EcR and RXR. F11 cells are the hybrid of a rat embryonic day-13 primary cultured neuron with a mouse neuroblastoma NTG18. These cells are one of the best models for primary cultured neurons; they exhibit, without differentiation factor treatment, a number of characteristics of primary neurons, including generation of action potentials. The cells were grown in Ham's F-12 plus 18% fetal bovine serum (FBS) and antibiotics (1% penicillin and streptomycin). F11 cells were the kind gift of I. Nishimoto, Department of Pharmacology and Neurosciences, KEIO University School of Medicine, Shinanomachi, Tokyo, Japan (Hashimoto et al., 2002).

2.2.2 Cell transfection

Cells were dissociated with incubations in Trypsin solution for 2 min and seeded at $7*10^4$ cells/well in a 6-well plate and cultured in Ham's F-12 plus 18% FBS for over night. Cells were then transfected with DNA using the FuGENE 6 reagent at a ratio (v/v) of 2:1 (according manufacturer protocol) and incubated overnight.

DNA plasmids used in transfections are summarised in Table 2.1. The propagation and isolations of DNA plasmids are described in section 2.3.

CBP plasmids	Description	Reference/Source
pGal4-(1-147)	The pGal(1-147) plasmid encoding the DNA binding domain of the yeast protein Gal4 was digested with HindIII and XbaI, and the DNA fragment encoding Gal4 amino acods 1-147 subcloned into the plasmid Rc/RSV (Invitrogen). This plasmid, Rc/RSV Gal-(1-147), was used as a basis to construct the remaining chimeric proteins.	J. Chrivia, Vollum Institute, Oregon Health Sciences University, Portland, Oregon 97201, USA (Swope et al., 1996)
pGal4-CBP-(1-460)		
pGal4-CBP-(721-1679)		
pGal4-CBP-(1678-1843)		
pGal4-CBP-(1844-1956)		
pGal4-CBP-(1961-2039)		
pGal4-CBP-(2060-2179)		
pGal4-CBP-(2173-2288)		
pGal4-CBP-(2288-2441)		
pGal4-CBP-(Full length)		
5xgal4-E2B TATA luciferase	Contains five copies of Gal4-binding site upstream of the TATA box from the viral E1B promoter, which is linked to the coding sequence of firefly luciferase. This plasmid was prepered by insertion of a HindIII-BamHI DNA E1B TATA box from G_5CAT into a plasmid containing the luciferase coding sequence.	R.A Maurer, Department of Cell and Developmental Biology, Oregon health Sciences University, Oregon, USA (Sun et al., 1994)
CBP full length	Full length mouse CBP (2442 amino acids) was cloned into Rc/RSV (Invitrogen).	Y.Z. Liu, Institute of Child Health, London, UK.
CBP antisense	Constructed by cloning CBP full-length (1-7326) into the BamHI site of bluescript SK (Stratagene) in the reversed orientation and subsequently cut the insert with NotI and SalI and inserting it into a pBI-G expression vector.	R.J. Gusterson, Institute of Child Health, London, UK.
Gal4-CBP HAT WT	CBP was cloned into HindIII/NotI site of pcDNA3 (Invitrogene) from pRc/RSV mCBP.	R. Eckner, Institute for Molecular Biology, University of Zurich, Winterthurerstrasse, Zurich, Switzerland (Bordoli et al., 2001b)
Gal4-CBP HAT WY	Amino acids 1503–1504 replaced by alanine and serine	

p300 plasmids	Description	Reference/Source
pGal4-p300	The pGal4-p300 fusion vector was made by inserting Gal4 1-147 sequwnce into p300 cDNA at the position for the 19th amino acid and subcloning the fusion unit into the pcDNA (Invitrogene). The p300 deletions were made from the pGal-p300 by digestion with restriction enzymes and re-ligation.	A. Giordano, Jefferson Cancer Institute and Department of Microbiology/Immunology and Pathology, Thomas Jefferson University, PA, USA (Yuan et al., 1996)
pGal4-p300-(1-743)		
pGal4-p300-(1242-1737)		
pGal4-p300-(964-1922)		
pGal4-p300-(1514-1922)		
pGal4-p300-(1737-2414)		
pGal4-p300-(1945-2414)		

Other plasmids	Description	Reference/Source
pIND WT PS1	PS cDNAs were digested with BamH I - EcoR I and subcloned into pIND plasmids	I. Nishimoto, Department of Pharmacology and Neurosciences, KEIO University School of Medicine, Shinanomachi, Tokyo, Japan (Hashimoto et al., 2002)
pIND M146L PS1		
pcDNA3 WT PS1	This plasmids contain PS cDNAs that were subcloned in pcDNA	Dr. G. Lévesque, Molecular and Human Genetics Unit, CHUQ-Pavillon St-François d'Assise, Canada
pcDNA3 Y115H PS1		
pcDNA3 M146L PS1		
pcDNA3 E318G PS1		
pcDNA3 L392V PS1		
c-fos-luciferase	A 447 bp fragment containing the human c-fos promoter (from −405 to +42 bp) was inserted into the pLucIAV vector upstream of the luciferase reporter gene	Dr. J.S Ramsdell, Center for Coastal Environmental Health and Biomolecular Research, NOAA-National Ocean Service, Charleston, SC, USA
TK-Renilla luciferase	This contains the Renilla luciferase gene under the control of the minimal thymidine kinase promoter	Promega UK. Southampton, United Kingdom

Table 2.1 DNA plasmids.

2.2.3 Stimulation of cells

PD 98059 (PD), a p42/p44 MAPK inhibitor, was dissolved in DMSO and used at a concentration of 25μM (Promega Corporation, Southampton, UK).

LY 294002 (LY), a PI 3-kinase inhibitor, was dissolved in DMSO and used at a concentration of 50μM (Promega Corporation, Southampton, UK).

SB 203580 (SB), a p38 MAPK inhibitor, was dissolved in DMSO and used at a concentration of 10μM (Promega Corporation, Southampton, UK).

Ponasterone A, which was employed as an ecdysone analogue (EcD), was then added 8 hr after transfection and incubated overnight (Invitrogen Ltd, Paisley, UK).

2.2.4 siRNA

siGENOME Non-Targeting siRNA #1 was purchased from Thermo scientific (Chicago, USA).

Presenilin 1 mouse siRNA was purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA.

2.3 Propagation, purification and manipulation of plasmid DNA

All manipulations of bacterial cultures were carried out under sterile conditions. All media and glassware were autoclaved before use at 120°C, 10psi for 20 minutes, and sterile plasticware was used. Manipulations were carried out over a Bunsen burner flame to prevent contamination.

2.3.1 Transformation of E.coli

The E.coli strain XL1-blue was used for the propagation of plasmid DNA. XL1-blues were streaked on a plate of Luria Bertani (LB) Agar (1% (w/v) Bacto®-tryptone, 1% (w/v) NaCl, 0.5% (w/v) Bacto®-yeast extract, and 2% (w/v) Bacto®-Micro agar). 24 hours later a single colony was picked and used to inoculate 5mls of LB medium (1 % (w/v) Bacto®-tryptone, 1% (w/v) NaCI, 0.5% (w/v) Bacto®-yeast extract). This starter culture was grown overnight in an orbital shaker at 37°C, 200rpm.

In order to allow transformation, bacteria must be made competent to allow entry of plasmid DNA. 100µl of starter culture was used to inoculate 100ml of LB and cultured in the orbital shaker until the culture had an optical density 600nm of 0.4-0.5 units (between 4-6 hours). The cultures were then pelleted in sterile 50ml tubes by centrifugation at 2500g at 4°C for 10 minutes. The supernatant was then discarded and the pellets re-suspended in 5 ml ice cold 100mM $CaCl_2$ for 1 hour. The bacteria were then centrifuged as above and resuspended in 1ml ice cold 100mM $CaCl_2$ and incubated on ice for 1 hour prior to use.

For transformation, 100µl of competent cells were aliquoted into 1.5ml sterile microcentrifuge tubes, mixed with 1 µg of plasmid DNA and incubated on ice for 10 minutes. The cells were then heat shocked at 42°C for 90 seconds and cooled on ice for 2 minutes. 800µl of LB medium was added and the cells incubated in an orbital shaker for 1 hour. The cells were then centrifuged at 145g for 1 minute at 20°C. 800µl of supernatant was discarded and cells re-suspended in the remaining 100µl. The cell suspension was then spread onto LB agar plates containing the appropriate selectable marker. Ampicillin was used at a concentration of 50µg/ml and cells were spread onto agar plates that were incubated at 37°C overnight and then stored for up to one month at 4°C.

2.3.2 Large scale plasmid DNA extraction from E.coli

Qiagen-tip 100 plasmid maxi prep kits were used according to manufacturer's instructions to prepare plasmid DNA for transfections. 200ml of LB medium containing the appropriate selectable marker was inoculated with an E.coli colony containing the plasmid of interest, and grown up overnight in an orbital shaker at 37°C, 200rpm. Isolation of plasmid DNA was achieved by alkaline lysis of cells followed by purification of plasmid on the Qiagen resin, elution and precipitation in isopropanol. Precipitated DNA was centrifuged at 12000g for 30 minutes at 4°C, washed in 70% ethanol, dried and re-suspended in 50µl sterile water. Details of this technique can be found in the Qiagen plasmid purification handbook.

DNA was quantified by spectrophotometry, reading the absorbance at 260nm and 280nm wavelengths. DNA concentrations were determined by the 260nm reading.

An absorbance of 1 indicated 50μg/ml of double stranded DNA. Measurement of absorbance at 280nm gave an estimation of the amount of protein contaminants present in the sample since the ratio of A_{260}/ A_{280} should be 1.7 for a pure DNA sample. Average yields using this technique were 100μg DNA. Plasmid stocks were stored at -20°C.

2.3.3 Small scale plasmid DNA extraction from E. coli

Small scale plasmid QIAprep Mini preps were used to purify DNA from several colonies to enable screening for the correct plasmid insert. 5ml of LB media containing the appropriate selectable marker was inoculated with a single colony and grown overnight in an orbital shaker at 37°C, 200rpm. The culture was transferred to a tube and centrifuged at 5000g for 10 minute (leaving 1ml of the culture at 4°C for future maxiprep). Pelleted cells were re-suspended, subjected to alkaline lysis and neutralised. The DNA was then absorbed onto a silica gel membrane, washed and eluted. Details of this technique can be found in the QIAprep® miniprep handbook.

2.3.4 Examination of DNA by restriction digest

Plasmid DNA was digested with restriction enzymes to characterise plasmid structure. 1 μg DNA was incubated with 10 units of enzyme, 1 μl enzyme buffer in a total volume of 10μl, made up with ddHzO at 37°C for 1 hour. The enzyme buffer and enzyme concentration varies for different enzymes and was carried out as specified by the Promega technical information sheets.

Agarose gel electrophoresis was carried out to examine the size of the plasmid fragment generated by restriction digests. 1% agarose (w/v) was dissolved in lxTAE (0.04M Tris, 0.02M sodium acetate, lmM EDTA, adjusted to pH 8.3 with HCl) and melted in a microwave oven. After cooling for 5-10 minutes, ethidium bromide was added to a final concentration of 0.5μg/ml, and the gel was poured into a gel casting tray containing a suitable comb. Once set, the comb was removed and the gel placed in an electrophoresis tank containing lxTAE. DNA loading buffer 6 x stock (0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol in water) was added to DNA to a final 1x concentration. Samples were loaded alongside a lkb ladder and run at approximately 10 V/cm until the loading dye had travelled a sufficient distance through the gel. DNA bands were observed and photographed on a Syngene UV light box.

2.4 Analysis of protein Levels

2.4.1 Western blotting

Cells were washed in PBS, harvested by scraping in 1 x SDS gel loading buffer (50mM Tris, 100mM Dithiotheitol, 2% SDS, 0.1 % bromophenol blue, 10% glycerol) and heated to 100cC for 5 minutes. Polyacrylamide gel electrophoresis was carried out using a 10% polyacrylamide gel (10% (v/v) acrylamide, 375mM Tris-HCI pH 8.8, 0.1 % (w/v) SDS, 0.05% (w/v) ammonium persulphate, 0.05% (w/v) N,N,N,'N'-tetramethylethylenediamine (TEMED)), with a 5% stacking gel (5% acrylamide, 12SmM Tris-HCI pH 6.8, 0.1 % SDS, 0.1 % ammonium persulpliate, 0.1 % TEMED). A molecular weight rainbow marker was run to identify the size of protein bands. The gel was run in Tris-glycine running buffer (25mM Tris, 250mM glycine (pH 8.3), 0.1 % SDS) at 30mA for 4-6 hours, or until the dye front was within 3cm of the bottom of the gel. Protein was transferred from the gel to a Hybond-c membrane overnight in a Trans-Blot transfer cell in blot buffer (l92mM glycine 20% (w/v) methanol, 25mM Tris-HCI pH 8).

The membrane was then probed on a shaking platform at room temperature as follows: non-specific sites were blocked by incubation in block buffer (5% Marvel (skimmed milk powder) in PBS) for 1 hour. The primary antibody was then added (diluted in block buffer) and incubated over night at 4°C. The membrane was then 5x washed in wash buffer (1 % Marvel, 1 % Tween 20 in PBS) for 5 minutes. Secondary antibody (HRP conjugate, in block buffer) was

incubated for 1 hour at RT. The membrane was then 5x washed in wash buffer for 5 minutes per wash.

ECL was used according to manufacturer's instructions to visualise labelled bands by exposure to photographic film. The membrane was stripped in O.2M glycine pH 2.9 for 15 minutes and re-probed with an antibody to actin to check for equal protein loading. Bands were quantified by densitometry on a BioRad GS-800 densitometer. Antibodies and conditions for use are shown in Table 2.2.

Primary antibody	**Dilution**	**Secondary antibody**	**Dilution**
CBP (C-1) monoclonal	1:50	Anti-Mouse Ig HRP	1:1000
PS1 polyclonal	1:1000	Anti-Rabbit Ig HRP	1:1000
Actin (1-19) monoclonal	1:2000	Anti-Goat Ig HRP	1:2000
Histone H4 polyclonal	1:1000	Anti-Rabbit Ig HRP	1:1000
Acetyl-Histone H4 polyclonal	1:1000	Anti-Rabbit Ig HRP	1:1000
GAL4 (DNA binding domain)	1:200	Anti-Rabbit Ig HRP	1:1000

Table 2.2 Antibody Conditions for western blots and immunofluorescence.

2.4.2 Confocal immunofluorescence

Cells were washed in PBS and were fixed in by adding –20°C methanol on ice for 10 minutes. Cells were then washed in PBS, and incubated in PBS 3% BSA for

blocking (3% BSA, 0.05% sodium azide in PBS) to block non-specific sites of antibody adsorption. Samples were stored at 4°C.

Primary CBP antibody was added to each sample for over night at 4°C, washed in PBS and non-specific sites blocked in block buffer for 15 minutes at room temperature. Secondary anti-mouse IgGl: RPE and DAPI stain (0.5 μg/ml) were added for 1 hour at room temperature (kept in the dark). Cells were washed in PBS and analyzed using Leica TCS SPE confocal microscop, which is capable of producing large xy and z montages.

2.4.3 Protein Assay

The amount of protein in the samples was quantified using the bicinchoninic (BCA) Protein Assay Reagent kit according to the manufacturer's instructions. This method uses colorimetric detection combining the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium with the highly sensitive colorimetric detection of cuprous cation using a reagent containing BCA. Diluted bovine serum albumin (BSA) standards were prepared and the cell lysate diluted using l0μl cell lysate and 40μ1 ddHzO. 50μ1 standards and diluted cell lysates ,were added to a 96 well microplate, 200μ1 BCA working reagent added to each well and the plate incubated at 37°C for 1 hour. The absorbance was measured at 560nm on a plate reader and the protein concentration calculated from the standard curve.

2.5 Assessment of promoter activity

100µl of 1x reporter lysis buffer was added to each well of cultured cells (based on 6 well plate) cells scraped with a rubber policeman and transferred to sterile 1.5ml microcentrifuge tubes. Samples centrifuged at l2000g for 1 minute to pellet cellular debris and the supernatant was transferred to sterile 1.5ml microcentrifuge tubes. Cell extracts were used to measure reporter construct activity.

2.5.1 Luciferase assay

The amount of luciferase produced by luciferase reporter constructs was quantified using a Luciferase Reporter 1000 assay system, according to the manufacturer's instructions. 50µl of cell extract was mixed with 100µl luciferase reagent and read for 20 seconds in a luminometer after a 5 second delay. This value represents the amount of firefly luciferase present in the sample. Transfections were equalised by measuring *Renilla* luciferase activity using the same procedure and this value was subsequently used to normalise values obtained in the firefly assay.

2.6 Animals

Hemizygous transgenic (HuAPP695SWE)2576 mice expressing mutant human APP (K670N,M671L)(Hsiao et al., 1996) were crossed with hemizygous PS1 mice that express mutant human PS1 (M146V; line 6.2)(Duff et al., 1996). The offspring, double-transgenic mice overexpressing APP/PS1, were compared with their WT littermates so that age and background strain were comparable. To identify the genotype of the animals, we used DNA extracted from tail tissue(Duff et al., 1996; Hsiao et al., 1996).

2.6.1 Drug administration.

Three-month-old APP/PS1 and WT mice were evenly separated into 4 groups: APP/PS1 mice treated with vehicle, APP/PS1 mice treated with TSA, WT mice treated with vehicle, and WT mice treated with TSA. For assessment of the short-term effects of TSA, the drug was given at a concentration of 2μgr per gram of body weight via i.p. injection 2 hours minutes before the behavioral tests. Finally, the animals were sacrificed for western blot analysis.

2.6.2 Measurement of LTP

Mice were decapitated, and their hippocampi were removed. Transverse hippocampal slices of a thickness of 400 μm were made on a tissue chopper and transferred to an interface chamber, where they were maintained at 29°C. They were perfused (1–3 ml/min) with saline solution (124.0 mM NaCl/4.4 mM KCl/1.0 mM Na2HPO4/25.0 mM NaHCO3/2.0 CaCL2/2.0 mM MgSO4/10 mM glucose) continuously bubbled with 95% O2 and 5% CO2. Slices were permitted

to recover for at least 90 min before recording. A concentric bipolar platinum-iridium stimulation electrode and a low-resistance glass recording microelectrode filled with saline solution (5 mΩ resistance) were placed in CA1 stratum radiatum to record the extracellular field excitatory postsynaptic potential (fEPSP). An input–output curve was used to set the baseline fEPSP at ≈35% of maximal slope. Baseline stimulation was delivered every minute (0.01-ms duration pulses) for 15 min before beginning the experiment to assure stability of the response. TSA (1.65 μM) or vehicle was added (in 0.1% DMSO) to perfused slices for 30 min in interleaved experiments. LTP was induced by using θ-burst stimulation (4 pulses at 100 Hz, with the bursts repeated at 5 Hz and each tetanus including three 10-burst trains separated by 15 s). Responses were recorded for 1 h after tetanization.

2.6.3 Contextual fear conditioning

Our conditioning chamber was located inside a sound-attenuating box (72 cm × 51 cm × 48 cm). A clear Plexiglas window (2 cm × 12 cm × 20 cm) allowed the experimenter to film the mouse performance with a camera placed on a tripod and connected to Freezeframe software (MED Associates Inc.). To provide background white noise (72 dB), a single computer fan was installed in 1 of the sides of the sound-attenuating chamber. The conditioning chamber (33 cm × 20 cm × 22 cm) was made of transparent Plexiglas on 2 sides and metal on the other 2. One of the metal sides had a speaker and the other had a 24-V light. The chamber had a 36-bar insulated shock grid floor. The floor was removable and after each use we cleaned it with 75% ethanol and then with water. Only 1 animal at a time was present in the experimentation room. The other mice remained in their home cages.

During the contextual conditioning experiment, mice were placed in the conditioning chamber for 2 minutes. In the last 2 seconds of the 2 minutes, mice were given a foot shock (US) of 0.50 mA for 2 seconds through the bars of the floor. After the US, the mice were left in the conditioning chamber for another 30 seconds and then were placed back in their home cages. "Freezing" behavior, defined as the absence of all movement except for that necessitated by breathing, was assigned scores using Freezeview software (MED Associates Inc.). For evaluation of contextual fear learning, freezing was measured for 5 (consecutive) minutes in the chamber in which the mice were trained at 24 hours after training.

2.7 Statistical Analysis

Where only two comparisons were required in a data set, student's t-tests were used to test for a significant difference between means, with a P value of less than 0.05 taken as significant. Two sample t-tests assuming unequal variance were performed using SPSS.

Where multiple comparisons were required between treatment groups, single factor analysis of variance (ANOVA) was used to look for differences between treatments. Single factor ANOVA was carried out using SPSS and a P value of less than 0.05 was taken as significant. t-tests are not recommended for multiple comparisons as the risk of obtaining a type I error (i.e. a false positive) is increased as more tests are performed. The Bonferroni test is a modified t-test where the P value is multiplied by the number of tests performed on the same data. This makes the chance of achieving significance less likely, and thus reduces the chance of type I errors. When ANOVA showed significant differences between groups, the Bonferroni method was performed post-hoc to test for significant difference between specific treatments. For LTP experiments the data analysis was performed with a factorial ANOVA with post hoc correction. The results were expressed as mean ± SEM. Significance was determined at the level of P less than 0.05.

Chapter 3

3 THE EFFECTS OF PS1 ON CBP

3.1 Introduction

As mentioned in section 1.8, AD is an age-related neurodegenerative disorder that is associated with the deterioration of memory and a progressive cognitive decline with the formation of neurofibrillary tangles and amyloid plaques. Three AD associated genes, APP, PS1 and PS2, were found to be the primary cause of the FAD(Kang et al., 1987; Levy-Lahad et al., 1995; Sherrington et al., 1995). PS1 is a part of the γ-secretase complex which is responsible for the cleaving of APP, leading to the increased production of Aβ42 peptide suggesting a toxic gain-of-function pathogenic mechanism.

As described in section 1.6, intensive studies of the mechanisms underlying memory formation have defined central roles for CRE-dependent gene expression, which is mediated by the transcription factor CREB and its coactivator CBP. CBP was found to acetylate histones and to form a bridge between the CREB-CBP complex and the basal transcriptional machinery resulting in transcriptional activity (Victor et al., 2002).

Previous investigations, as described in section 1.9, of normal presenilin function revealed a contradictory role of PS1-CBP in neurodegeneration. Thus, PS 1 and 2 conditional double knockout mice showed reductions in CBP levels and in CRE-dependent gene expression in the cerebral cortex, which is likely to contribute to subsequent neuronal degeneration(Saura et al., 2004) 17. In contrast, it has been shown that wild type PS1 cleaves the transmembrane protein N-cadherin to release an intracellular fragment. In turn, this fragment has been shown to bind to

CBP and promote rapid proteasomal degradation(Marambaud et al., 2003). Moreover, it has been shown that the mutant forms of PS1 found in familial Alzheimer's disease (FAD) cannot cleave N-cadherin and therefore do not promote the degradation of CBP. This suggests that, in contradiction to Saura *et al*, the mutation in PS1 would result in enhanced levels of CBP and that CBP over-expression is toxic in certain conditions.

In view of this contradictory literature, we began to investigate the potential role of CBP in Alzheimer's disease by studying its activity and the effects of manipulating its expression in cells containing wild type or mutant forms of the proteins that are mutated in the familial forms of Alzheimer's disease (FAD). To investigate the role of CBP we used a neural cell line (F11), which allows the inducible over-expression of wild type or mutated PS1(Hashimoto et al., 2002). In addition, in order to measure CBP activity, we used plasmid constructs in which CBP is linked to the DNA-binding domain of the yeast Gal-4 transcription factor and which therefore allows constitutive recruitment of CBP to the DNA so that its ability to stimulate transcription can be directly measured (Fig 3.1). We have previously used this system to demonstrate, for example, that NGF stimulates CBP activity in neuronal cells(Liu et al., 1998).

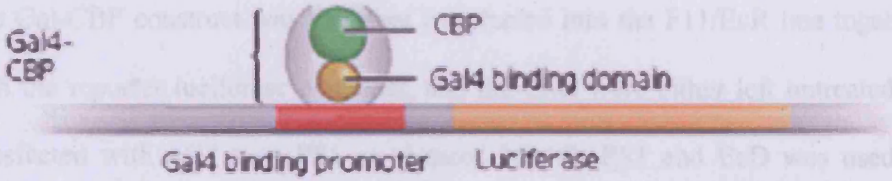

Figure 3.1 CBP/Gal4 Chimera

CBP/Gal4 hybrid and a reporter plasmid in which luciferase expression is driven by a Gal4 yeast promoter.

3.2 The effect of WT/mutant PS1 on the transcriptional activity of CBP

The Gal-CBP construct was therefore transfected into the F11/EcR line together with the reporter luciferase construct, and the cells were either left untreated or transfected with wild type PS1 or mutated M146L PS1 and EcD was used to induce PS1 expression. As mentioned in section 2.2, F11 are one of the best models for primary cultured neurons; they exhibit, without differentiation factor treatment, a number of characteristics of primary neurons, including generation of action potentials, synthesis of neurotransmitters, expression of neuropeptide receptors, and voltage-gated calcium channels(Hashimoto et al., 2002; Jow et al., 2006).

In these experiments, a significant enhancement of CBP driven promoter activity was observed in the cells transfected with constructs containing wild type PS1 (Fig. 3.2). Compared to the control, wild type PS1 produced no enhanced activity when a construct containing the isolated DNA binding domain of Gal 4 without CBP was transfected (Fig. 3.3), indicating that wild type PS1 enhances the transcriptional stimulating ability of CBP. Remarkably, the mutant form of PS1 produced virtually no enhancement in promoter activity, showing similar activity levels to the untreated cells (Fig. 3.2). These results demonstrate that the mutant form of PS1, unlike wild type PS1, has no ability to activate CBP (Fig, 3.2). In addition, virtually no enhancement in CBP activity was observed, in the absence of PS1 transfection, when cells were treated with EcD, showing that EcD itself has no effect on CBP activity (Fig. 3.4). These experiments were routinely done with the addition of EcD to induce PS1 expression. Only minor enhancement in

CBP activity was observed, in the absence of EcD, when the cells were transfected with WT PS1 (Fig. 3.4).

As mentioned in section 1.8, there are 147 different PS1 mutants. It was therefore decided to examine if other PS1 mutations would lead to a reduction in CBP activity as demonstrated in the case of PS1 M146L mutant. Moreover, it was decided not to conduct this experiment in the EcD inducible system and to use a pcDNA3 expression vector in order to avoid any effect EcD might have on CBP activity and to show the general effect of PS1 mutants.

The Gal-CBP construct was therefore transfected into the F11/EcR line together with the reporter luciferase construct, and the cells were either transfected with empty pcDNA3 vector (control)/pcDNA3 wild type PS1 or PS1 mutants (Y115H, M146L, E318G and L392V all in pcDNA3 vector). As in the experiments above, a significant enhancement of CBP driven promoter activity was observed in the cells transfected with the construct containing wild type PS1 (Fig. 3.5). Interestingly, PS1 N-terminal mutants (Y115H and M146L) produced no enhanced activity and showed similar activity levels to the control cells (Fig. 3.5). Moreover, PS1 C-terminal mutants (E318G and L392V) showed a reduction in promoter activity compared to the pcDNA3 vector control. These results demonstrate that not only do PS1 mutants, unlike wild type PS1, have no ability to stimulate CBP transcriptional activation but that C-terminal PS1 mutants actually reduce CBP activity (Fig. 3.5).

The increase of CBP driven promoter could be as a result of increase of CBP

levels, increase in CBP enzymatic activity or even both. It was therefore decided to examine if transfected Gal-CBP levels change during the Gal-CBP experiments. Western blots analysis of extracts prepared from F11 cells transfected with Gal-CBP construct together with empty pcDNA3 vector (control)/pcDNA3 WT PS1 or pcDNA PS1-M146L, using Gal4 DNA binding domain antibody, showed no difference in levels of Gal-CBP between the three different groups (Fig 3.6).

Additionally, it was decided to measure endogenous CBP levels in order to determine if, in addition to CBP changes in enzymatic activity, a decrease in CBP levels is involved in the observed reduction in CBP driven promoter activity when PS1-M146L mutant is transfected. Western blot analysis of extracts prepared from F11 cells transfected with pcDNA3 WT PS1 revealed, compared to control, a significant increase in levels of endogenous CBP (Fig 3.7). In addition, an increase in CBP endogenous levels was noted when the cells were transfected with pcDNA3 PS1-M146L, however, these levels were still significantly lower when compared to cells transfected with pcDNA3 WT-PS1 (Fig 3.7).

Therefore, while wild type PS1 increases endogenous CBP levels more than PS1-M146L mutant, it does not account for our Ga4-CBP results. Gal4-CBP activity changes cannot be explained by changes in Gal4-CBP level since Gal4-CBP levels stay the same during the luciferase experiments. Consequently effects on Gal4 promoter must be due to changes in the activity of CBP, enhancing its ability to activate transcription.

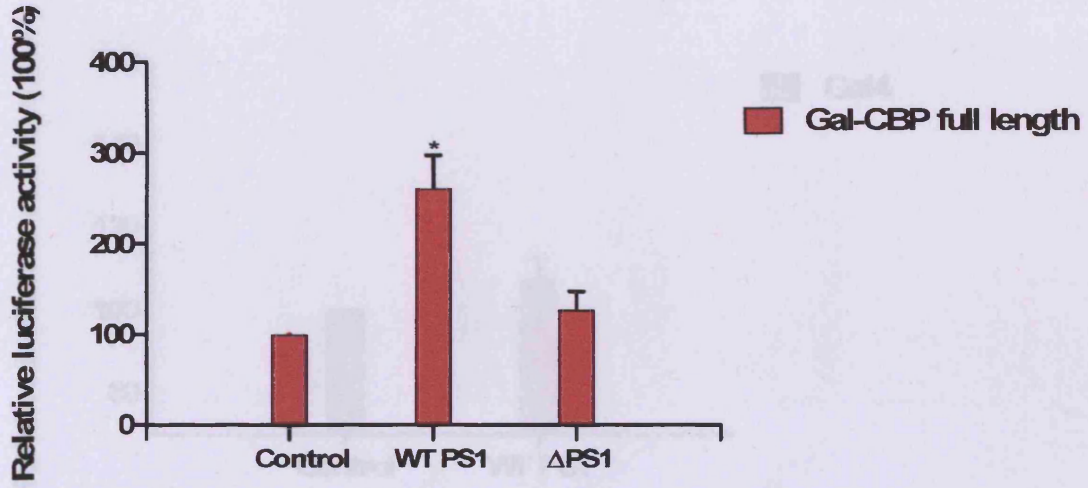

Figure 3.2 Effect of wild type or mutated PS1 on the transcriptional activity of CBP in F11 cells.

Luciferase assay results of co-transfecting F11 cells with 2 μg of the Gal4-E1B TATA-luciferase, 2 μg of the Gal-CBP (full length)-in the presence or absence of with wild type or mutated PS1 (1 μg). Values are expressed relative to the level of luciferase in control, neural cells transfected with Gal-luciferase and Gal-CBP (set at 100%) and are the means of three independent transfection experiments whose standard error is indicated by the bars. *, $p < 0.05$ versus control.

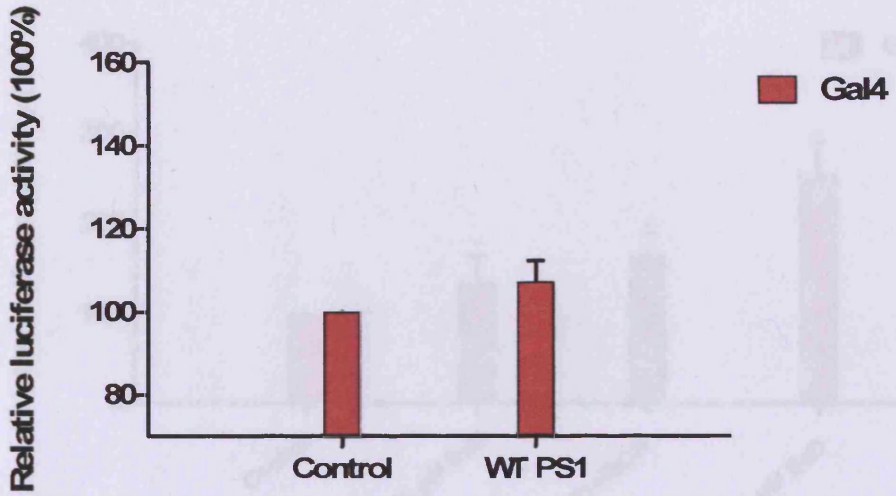

Figure 3.3 Effect of wild type PS1 on the transcriptional activity of Gal-4 in F11 cells.

Luciferase assay results of co-transfecting F11 cells with 2 μg of the Gal4-E1B TATA-luciferase, 2 μg of the Gal-4 in the presence or absence of with wild type PS1 (1 μg). Values are expressed relative to the level of luciferase in control, neural cells transfected with Gal-luciferase and Gal-4 (set at 100%) and are the means of three independent transfection experiments whose standard error is indicated by the bars. *, $p < 0.05$ versus control.

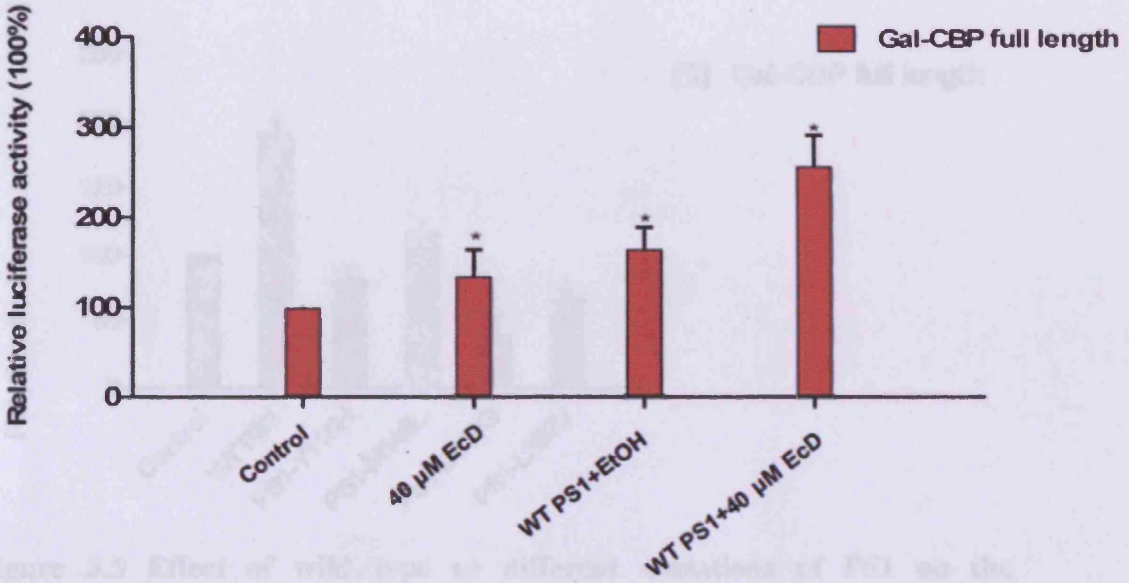

Figure 3.4 Effect of EcD and wild type PS1 on the transcriptional activity of CBP in F11 cells.

Luciferase assay results of co-transfecting F11 cells with 2 μg of the Gal4-E1B TATA-luciferase, 2 μg of the Gal-CBP (full length)-in the presence or absence of with wild type PS1 (1 μg) -in the presence or absence of EcD (40 μM). Values are expressed relative to the level of luciferase in control, neural cells transfected with Gal-luciferase and Gal-CBP (set at 100%) and are the means of three independent transfection experiments whose standard error is indicated by the bars. *, $p < 0.05$ versus control.

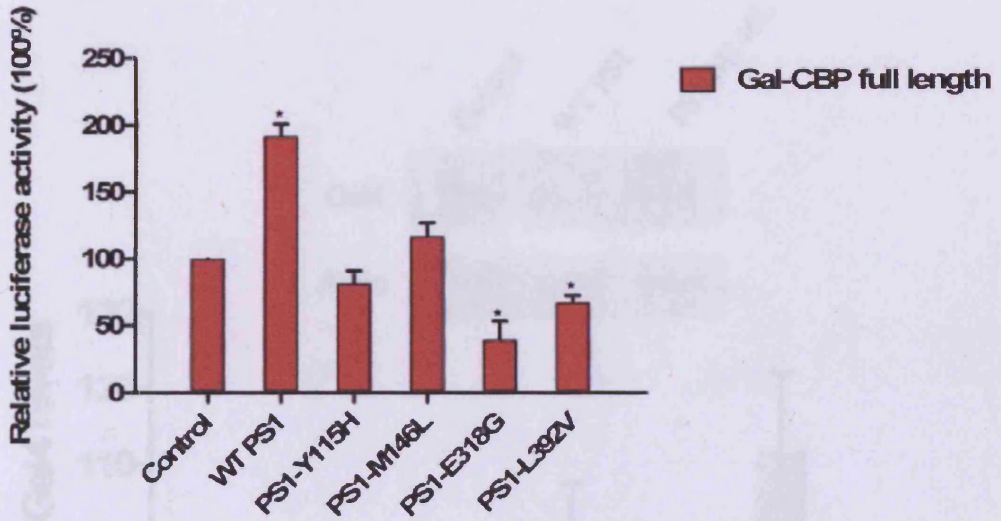

Figure 3.5 Effect of wild type or different mutations of PS1 on the transcriptional activity of CBP in F11 cells.

Luciferase assay results of co-transfecting F11 cells with 2 μg of the Gal4-E1B TATA-luciferase, 2 μg of the Gal-CBP (full length)-in the presence or absence of with PS1 wild type or one of PS1 mutants (Y115H, M146L, E318G and L392V) (1 μg). Values are expressed relative to the level of luciferase in control, neural cells transfected with Gal-luciferase and Gal-CBP (set at 100%) and are the means of three independent transfection experiments whose standard error is indicated by the bars. *, $p < 0.05$ versus control.

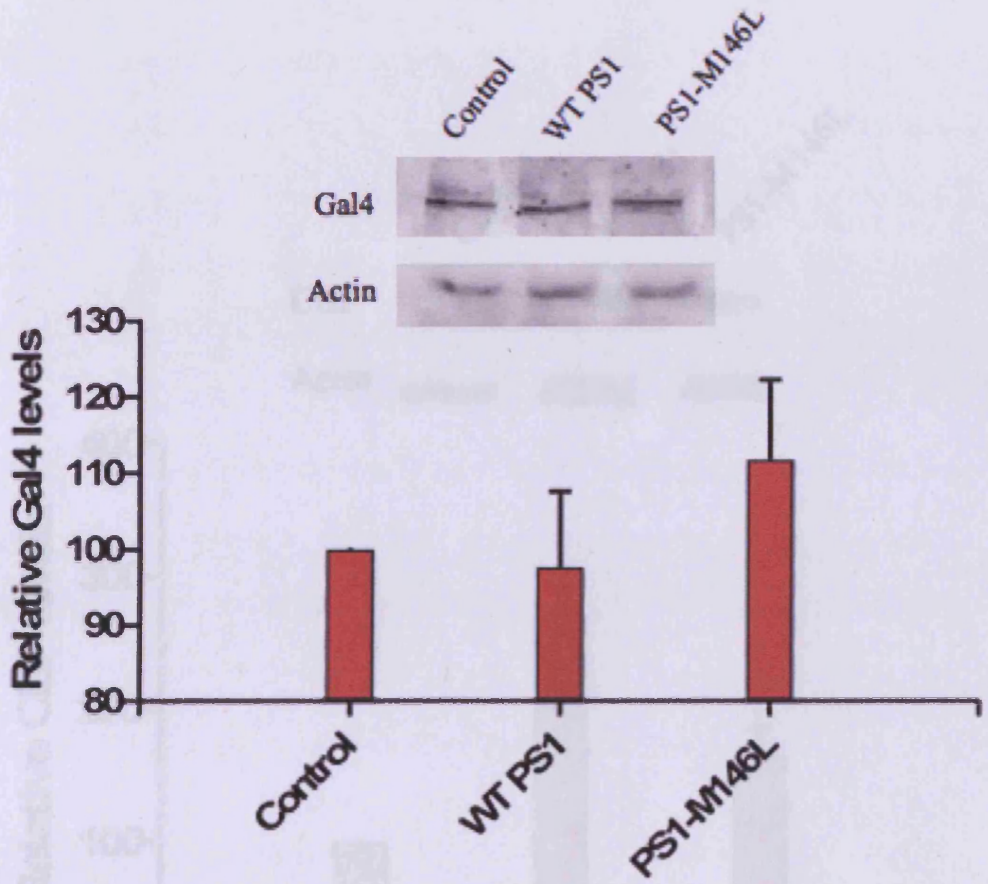

Figure 3.6 Effect of PS1 WT/mutant on transfected CBP levels

Expression of Gal4 in F11. Cells were transfected with Gal-CBP together with an empty pcDNA3 vector/pcDNA3 WT PS1/pcDNA3 PS1-M146L. Values are means of three independent transfection experiments whose standard error is indicated by the bars. $p > 0.05$ versus control. Results were normalized against the control. *, $p < 0.05$ versus control

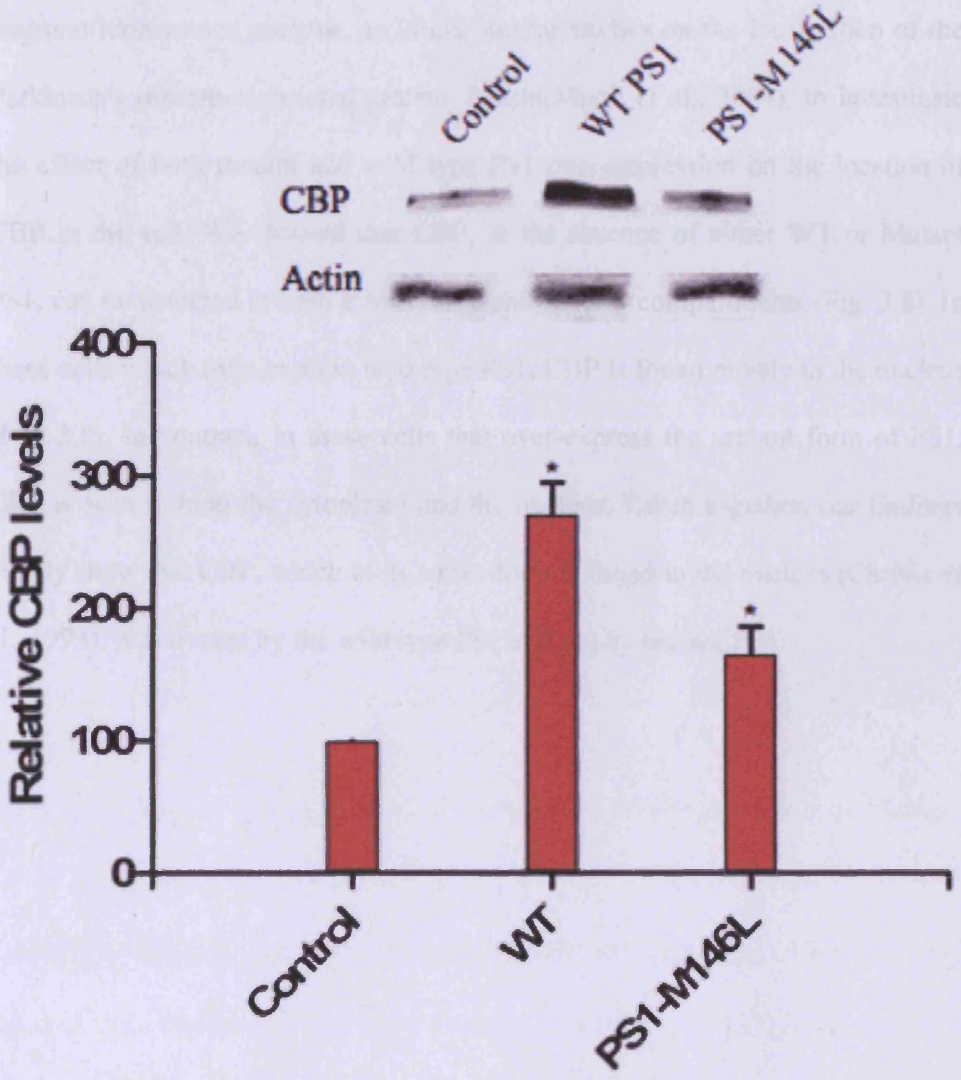

Figure 3.7 Effect of PS1 WT/mutant on endogenous CBP levels

Endogenous expression of CBP in F11 cells that were transfected either with an empty vector or PS1 WT/M146L in a pcDNA3 vector. Values are means of three independent transfection experiments whose standard error is indicated by the bars. $p > 0.05$ versus control. Results were normalized against the control. *, $p < 0.05$ versus control

3.3 Localisation of CBP during WT PS1 upregulation

To confirm our experiments with the WT and mutant PS1, we used confocal immunofluorescence analysis, as in our similar studies on the localisation of the Parkinson's disease-associated protein, Parkin(Muqit et al., 2004), to investigate the effect of both mutant and wild type PS1 over-expression on the location of CBP in the cell. We showed that CBP, in the absence of either WT or Mutant PS1, can be detected in both cytoplasmic and nuclear compartments (Fig. 3.8). In those cells which over-express wild type PS1, CBP is found mostly in the nucleus (Fig. 3.8). In contrast, in those cells that over-express the mutant form of PS1, CBP is seen in both the cytoplasm and the nucleus. Taken together, our findings clearly show that CBP, which in its active form is found in the nucleus (Chrivia et al., 1993), is activated by the wild type PS1 and not by mutant PS1.

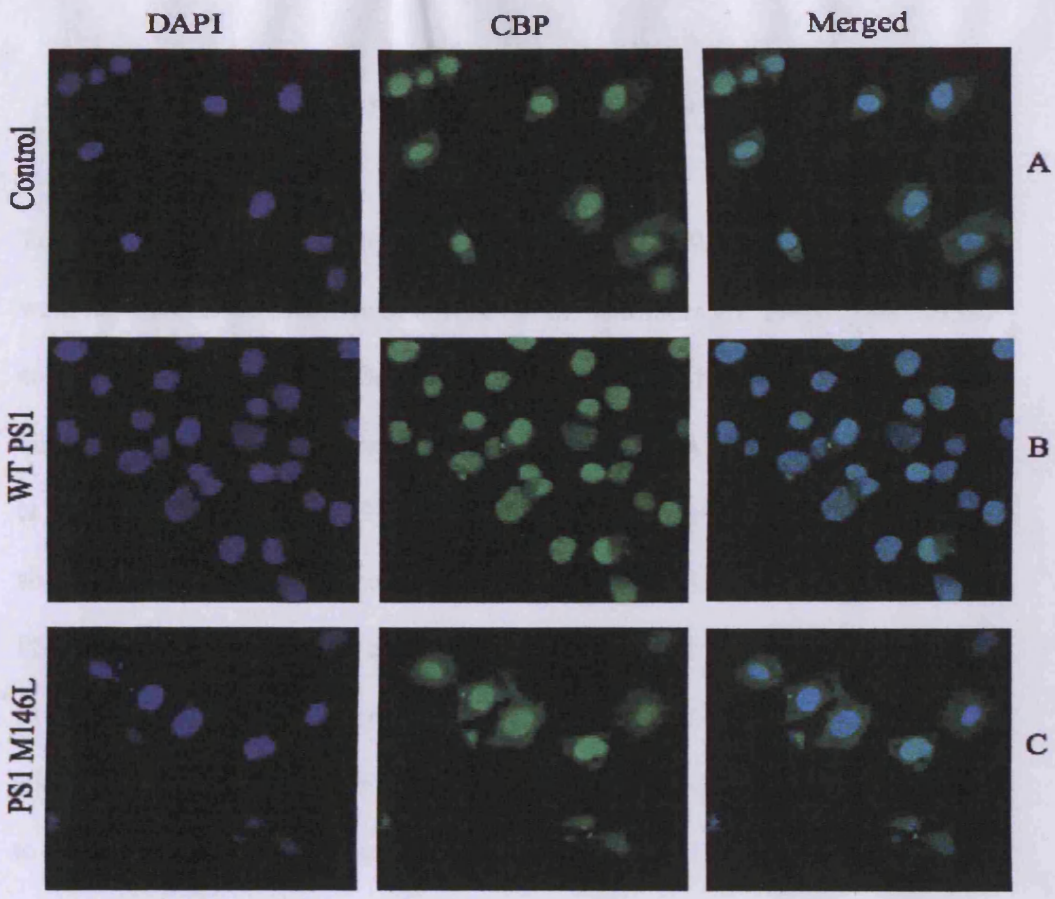

Figure 3.8 Effect of wild type and mutated PS1 on the cellular localizatio CBP in F11 cells.

Immunofluorescence results of F11 cells transfected with 1 μg of RSV-CBP (full length)-in the presence of wild type or mutated PS1 or pcDNA(1 μg). Cells v plated onto coverslips, fixed and prepared for indirect immunofluorescence. CBP protein was detected using the mouse monoclonal anti-CBP antibody (shown in green). Cells were counterstained with the Hoechst DNA dye (shown in blue). Both CBP and Hoechst staining patterns were merged. Digital images v captured using a Zeiss Axiocam camera. Representative fields are displayed. A) In control cells transfected with CBP and pcDNA, CBP was detected in the cytoplasm and the nucleus. B) In cells transfected with CBP and WT PS1, CBP was detected predominantly in the nucleus. C) In cells transfected with CBP and ΔPS1, CBP was detected both in the nucleus and cytoplasm.

3.4 Signalling pathways involved in the upregulation of CBP activity by PS1

To investigate the mechanisms mediating the effect of WT PS1, the experiments were repeated in the presence or absence of the inhibitors LY 294002, SB 203580 and PD 98059 which specifically inhibit the activity of PI 3-kinase, p38 MAP kinase and p42/p44 MAP kinase pathways respectively(Alessi et al., 1995; Vlahos et al., 1994; Young et al., 1997). In these experiments addition of PD resulted in a slight increase in the basal activity produced by the CBP in the absence of WT PS1 (Fig. 3.9A) whereas the addition of LY and SB did not show such an effect. In addition, the up-regulation of CBP activity by WT PS1 was prevented by addition of LY, SB and PD (Fig. 3.9B), resulting in a similar lack of CBP activity to that produced by the mutant form of PS1. In addition, CBP showed the same activity in the presence of the mutant form of PS1, with or without the inhibitors LY 294002, SB 203580 and PD 98059 (Fig. 3.10). This suggests therefore that in neuronal cells the ability of WT PS1 to stimulate the transcriptional activity of CBP involves the PI 3-kinase, p38 MAP kinase and p42/p44 MAPK pathways.

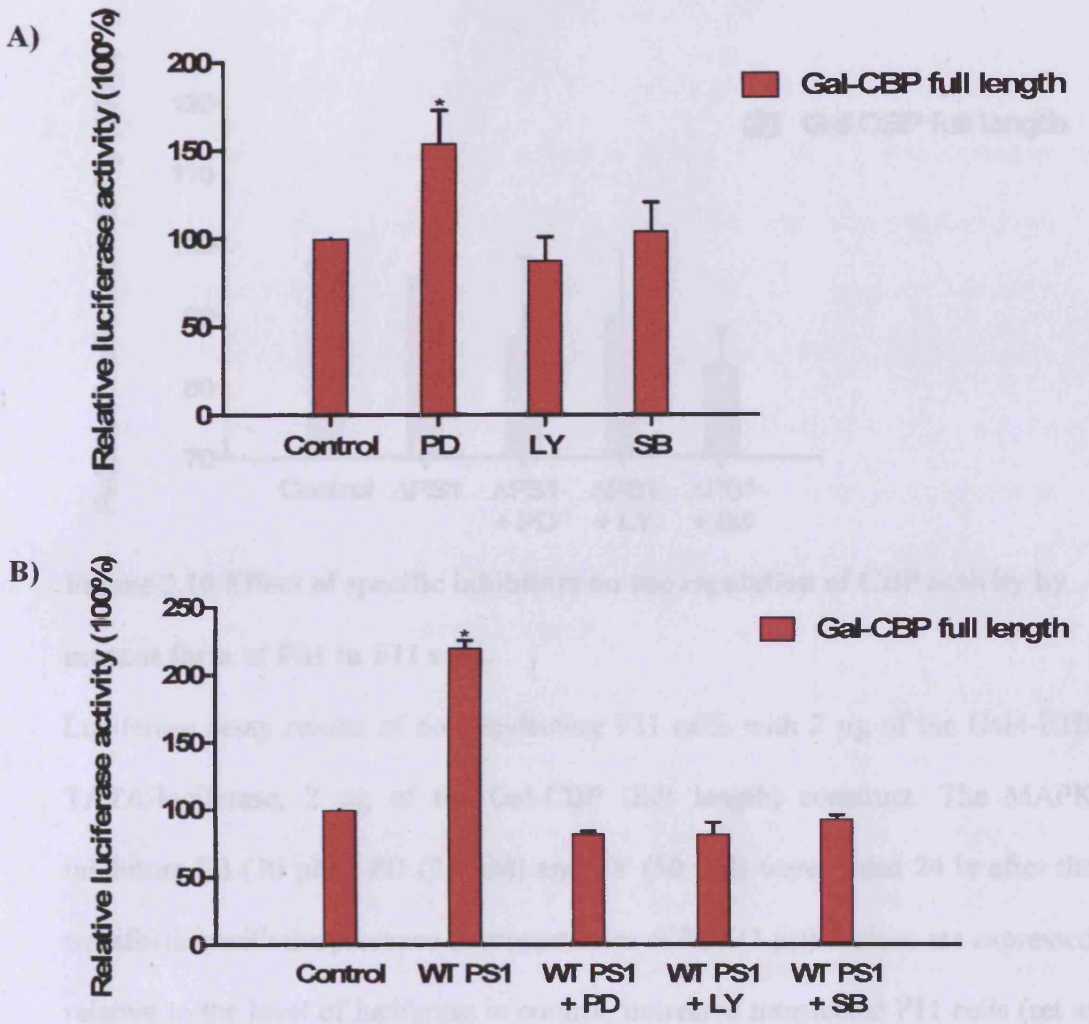

Figure 3.9 Effect of specific inhibitors on the up-regulation of CBP activity by WT PS1 in F11 cells.

Luciferase assay results of co-transfecting F11 cells with 2 μg of the Gal4-E1B TATA-luciferase, 2 μg of the Gal-CBP (full length) construct. The MAPK inhibitors SB (10 μM), PD (25 μM) and LY (50 μM) were added 24 hr after the transfection with (B) or without (A) the presence of WT PS1 (1 μg). Values are expressed relative to the level of luciferase in control, untreated transfected F11 cells (set at 100%) and are the means of three independent transfection experiments whose standard error is indicated by the bars. *, $p < 0.05$ versus control.

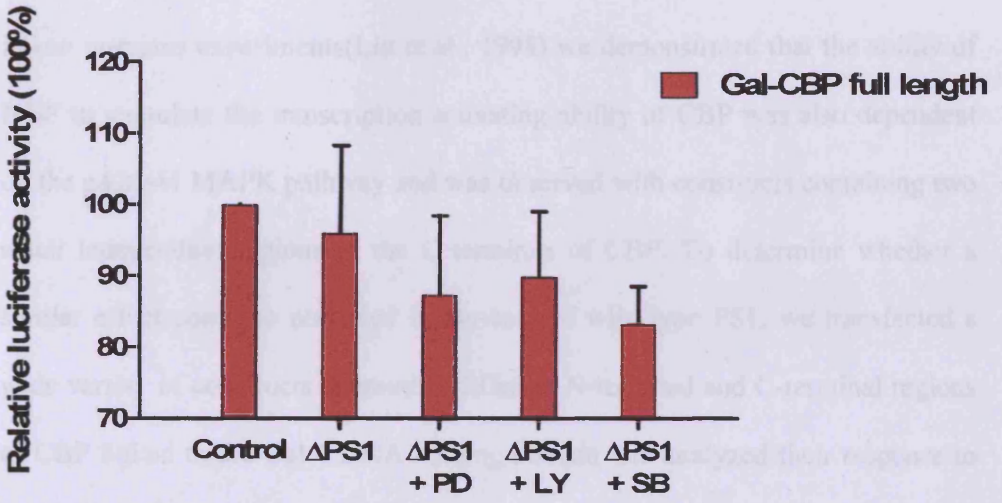

Figure 3.10 Effect of specific inhibitors on the regulation of CBP activity by mutant form of PS1 in F11 cells.

Luciferase assay results of co-transfecting F11 cells with 2 μg of the Gal4-E1B TATA-luciferase, 2 μg of the Gal-CBP (full length) construct. The MAPK inhibitors SB (10 μM), PD (25 μM) and LY (50 μM) were added 24 hr after the transfection with the presence of mutant form of PS1 (1 μg). Values are expressed relative to the level of luciferase in control, untreated transfected F11 cells (set at 100%) and are the means of three independent transfection experiments whose standard error is indicated by the bars. *, $p < 0.05$ versus control.

3.5 Regions of CBP involved in activation by WT PS1

In our previous experiments(Liu et al., 1998) we demonstrated that the ability of NGF to stimulate the transcription activating ability of CBP was also dependent on the p42/p44 MAPK pathway and was observed with constructs containing two small independent regions at the C terminus of CBP. To determine whether a similar effect could be observed in the case of wild type PS1, we transfected a wide variety of constructs containing different N-terminal and C-terminal regions of CBP linked to the Gal 4 DNA binding domain and analyzed their response to wild type PS1. In these experiments (Fig. 3.11) a strong response to wild type PS1 was observed with the construct containing the C-terminal region of CBP from amino acids 2173 through 2288. In addition, smaller responses of approximately 1.5-fold were observed with N-terminal regions 1-460, 721- 1679 and 1678-1843 (Fig. 3.11). However, a decreased activity in response to WT PS1 was shown with the construct containing the region of CBP from amino acids 1961-2039 (Fig. 3.11). This suggests that multiple regions at the N terminus of CBP can mediate the response to wild type PS1, but that a single short C terminus region produces the strongest effect.

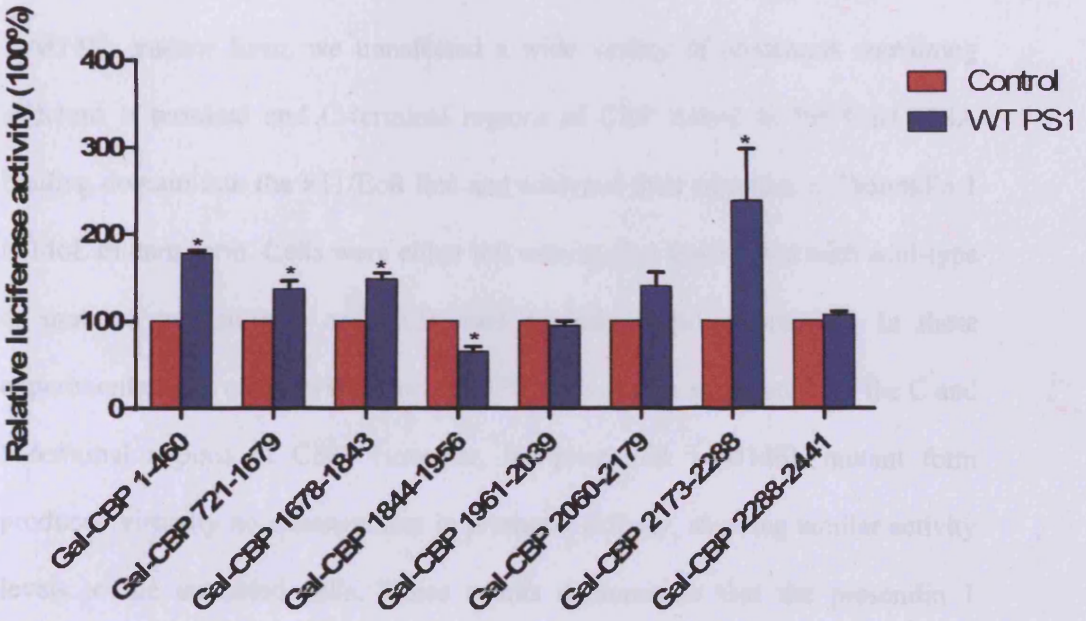

Figure 3.11. Effect of wild type PS1 on the transcriptional activity of different N-terminal and C-terminal regions of CBP in neural cell.

Luciferase assay results of co-transfecting F11 cells with with 2 μg of the Gal4-E1B TATA-luciferase together with 2 μg of the CBP C-terminal regions (amino acids 1961-2039, 2060-2179, 2173-2288, or 2288-2441) or CBP N-terminal regions (amino acids 1-460, 721-1679, 1678-1843 or 1844-1956) linked to Gal4 in the presence of WT PS1 (1 μg). In each case values are expressed relative to the level of luciferase in untreated F11 cells transfected with each construct (set at 100%) and are the means of three independent transfection experiments whose standard error is indicated by the bars. *, $p < 0.05$ versus the same construct without WT PS1.

To determine whether a similar effect could be observed in the case of presenilin 1 M146L mutant form, we transfected a wide variety of constructs containing different N-terminal and C-terminal regions of CBP linked to the Gal4 DNA binding domain into the F11/EcR line and analyzed their response to Presenilin 1 M146L mutant form. Cells were either left untreated or transfected with wild-type or mutated presenilin 1 and EcD used to induce PS1 expression. In these experiments, as in our previous one, WT PS1 was able to stimulate both the C and N-terminal regions of CBP. However, the presenilin 1 M146L mutant form produced virtually no enhancement in promoter activity, showing similar activity levels to the untreated cells. These results demonstrate that the presenilin 1 M146L mutant form, unlike wild-type Presenilin 1, has no ability to activate different regions of CBP (Fig. 3.12).

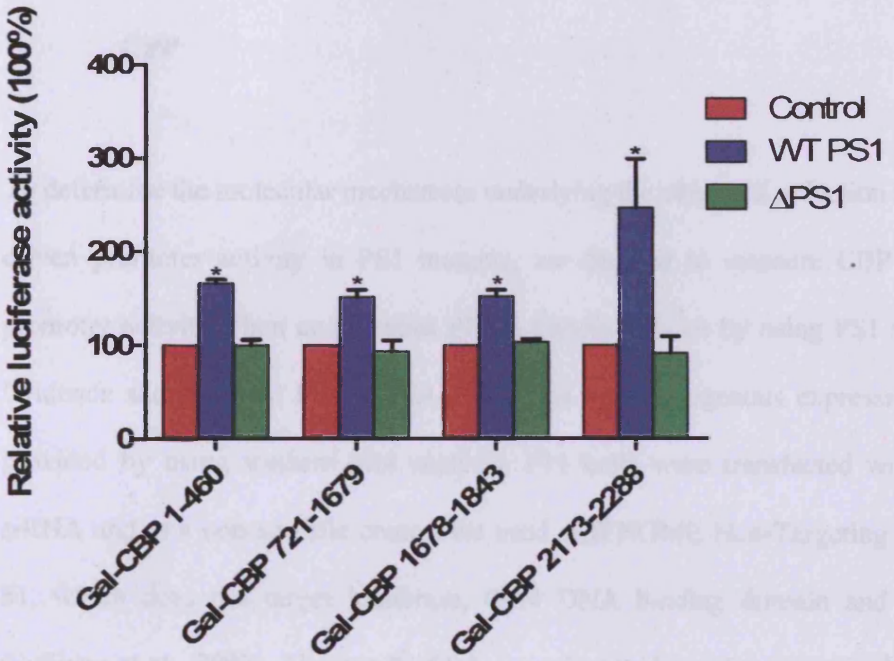

Figure 3.12 Effect of wild type and mutant forms of Presenilin 1 on the transcriptional activity of different N-terminal and C-terminal regions of CBP in neural cells.

Luciferase assay results of co-transfecting F11 cells with 2 μg of the Gal4-E1B TATA-luciferase together with 2 μg of the CBP C-terminal region (amino acids 2173–2288) or CBP N-terminal regions (amino acids 1–460, 721–1679, 1678–1843) linked to Gal4 in the presence of wild-type or mutant presenilin 1 (1 μg). Values are expressed relative to the level of luciferase in control (neural cells transfected with Gal-luciferase, different regions of Gal-CBP and 1 μg of empty vector) set at 100%. Values are the means of 3 independent transfection experiments whose s.e. is indicated by the bars. * $p<0.05$ vs control.

3.6 PS1 endogenous knockdown effect on the transcription activity of CBP

To determine the molecular mechanism underlying the observed reduction in CBP driven promoter activity in PS1 mutants, we decided to measure CBP driven promoter activity when endogenous PS1 is knocked down by using PS1 siRNA. Evidence showing that PS1 siRNA eliminates PS1 endogenous expression was provided by using western blot analysis. F11 cells were transfected with PS1 siRNA and as a non-specific control we used siGENOME Non-Targeting siRNA #1, which does not target luciferase, Gal4 DNA binding domain and renilla (Sullivan et al., 2007). The result of this experiment showed a dramatic decrease in levels of endogenous PS1 (Fig 3.13), confirming that the cells transfected with PS1 siRNA showed reduced PS1 expression. Moreover, we also confirmed the *in vivo* results of Saura *et al.*, which showed a decrease in CBP levels in PS cDKO mice (Saura et al., 2004). We were able to show that endogenous knockdown of PS1 leads to a decrease in endogenous CBP levels *in vitro* (Fig 3.13).

We then wanted to see if, in addition to a decrease in endogenous CBP, CBP enzymatic activity is also affected by the knockdown of PS1. The Gal-CBP construct was therefore transfected into the F11/EcR line together with the reporter luciferase construct, and the cells were either transfected with 1μg of siGENOME Non-Targeting siRNA #1 or PS1 siRNA. Compared to the control, PS1 siRNA transfected cells showed a decreased promoter activity, indicating that the knockdown of PS1 decreases the transcriptional stimulating ability of CBP (Fig 3.14). As mentioned in section 3.2, the decrease of CBP driven promoter could be as a result of decrease of CBP levels and/or decrease in CBP enzymatic

activity. It was therefore decided to examine if the decrease in CBP promoter activity is due to a decrease in CBP levels and/or a decrease in CBP enzymatic activity. Western blots Analysis of extracts prepared from F11 cells transfected with siRNA and Gal-CBP, using Gal4 DNA binding domain antibody, showed no significant difference in levels of Gal-CBP (Fig 3.15), suggesting that PS1 knockdown affects both CBP levels and CBP enzymatic activity.

Therefore, while PS1 knock down decreased endogenous CBP levels, it does not account for our Ga4-CBP results. Gal4-CBP activity changes cannot be explained by changes in Gal4-CBP level since Gal4-CBP levels stay the same during the luciferase experiments. Consequently effects on Gal4 promoter most be due to changes in activity of CBP, enhancing its ability to activate transcription.

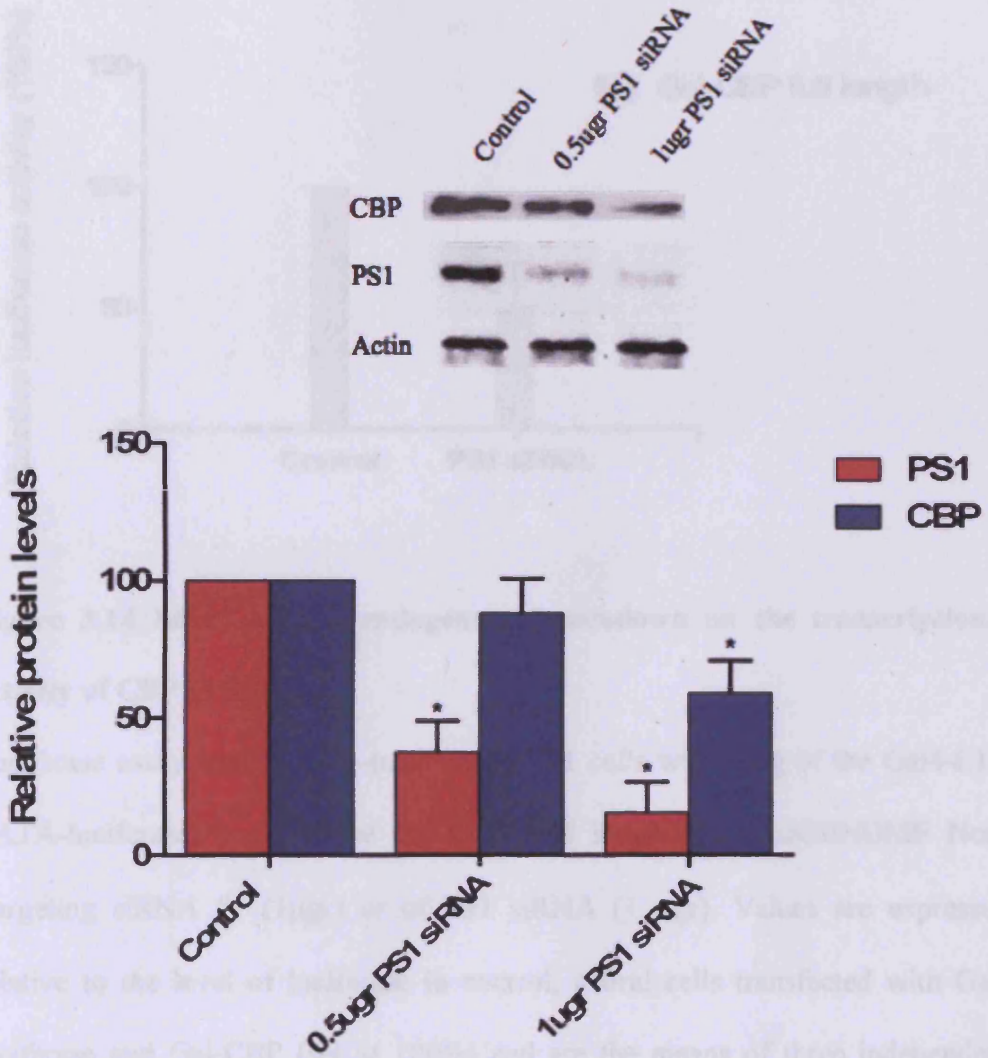

Figure 3.13 Effect of PS1 siRNA on endogenous PS1 and CBP levels

Endogenous expression of PS1 and CBP in F11 cells that were transfected either with siGENOME Non-Targeting siRNA #1 (1μgr) or siRNA. Values are means of three independent transfection experiments whose standard error is indicated by the bars. $p > 0.05$ versus control. Results were normalized against the control. *, $p < 0.05$ versus control

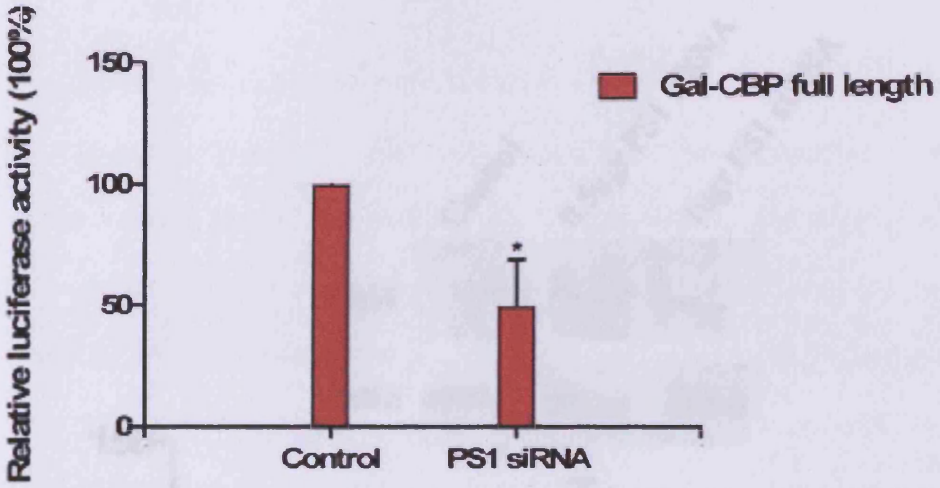

Figure 3.14 Effect of PS1 endogenous knockdown on the transcriptional activity of CBP in F11 cells.

Luciferase assay results of co-transfecting F11 cells with 2 µg of the Gal4-E1B TATA-luciferase, 2 µg of the Gal-CBP (full length)- with siGENOME Non-Targeting siRNA #1 (1µgr) or of PS1 siRNA (1 µgr). Values are expressed relative to the level of luciferase in control, neural cells transfected with Gal-luciferase and Gal-CBP (set at 100%) and are the means of three independent transfection experiments whose standard error is indicated by the bars. *, $p < 0.05$ versus control.

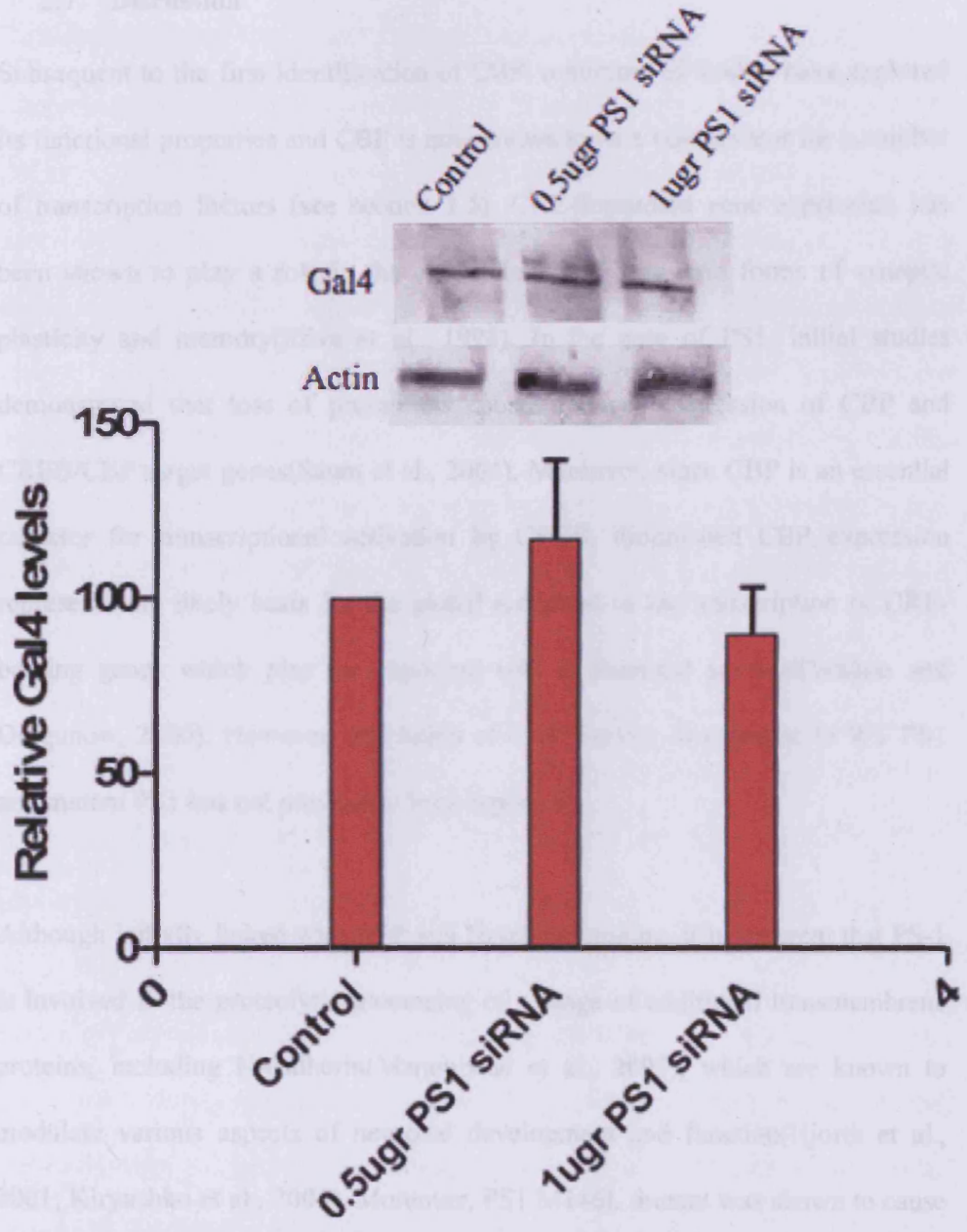

Figure 3.15 Effect of PS1 endogenous knockdown on transfected CBP levels

Expression of Gal4 in F11. cells that were transfected either with siGENOME Non-Targeting siRNA #1 (1μgr) or PS1 siRNA.Values are means of three independent transfection experiments whose standard error is indicated by the bars. $p > 0.05$ versus control. Results were normalized against the control. *, $p < 0.05$ versus control

3.7 Discussion

Subsequent to the first identification of CBP, a number of studies have explored its functional properties and CBP is now known to be a co-activator for a number of transcription factors (see section 1.5). CRE-dependent gene expression has been shown to play a role in the consolidation of long-term forms of synaptic plasticity and memory(Silva et al., 1998). In the case of PS1, initial studies demonstrated that loss of presenilins causes reduced expression of CBP and CREB/CBP target genes(Saura et al., 2004). Moreover, since CBP is an essential cofactor for transcriptional activation by CREB, diminished CBP expression represents the likely basis for the global reduction in the transcription of CRE-bearing genes which play an important role in neuronal survival(Walton and Dragunow, 2000). However, regulation of CBP activity in response to WT PS1 and mutant PS1 has not previously been reported.

Although initially linked with APP and Notch processing, it is apparent that PS-1 is involved in the proteolytic processing of a range of additional transmembrane proteins, including N-cadherin(Marambaud et al., 2003), which are known to modulate various aspects of neuronal development and function(Hjorth et al., 2001; Kiryushko et al., 2004). Moreover, PS1 M146L mutant was shown to cause neuronal cell death through to sensitivity to apoptosis(Hashimoto et al., 2002).

The data presented here add a new aspect to this by indicating that WT PS1, but not the mutant PS1 M146L form, can stimulate the transcriptional activation ability of CBP and increase CBP levels. To date, there are 170 known mutations in PS1, divided into N-terminal and C-terminal mutations, that are known to lead

to a familial form of Alzheimer's disease (Alzheimer Disease & Frontotemporal Dementia Mutation Database). As discussed in section 1.8, PS1 mutations are known to lead to enhance deposition of Aβ42 senile plaques in the brain(Gomez-Isla et al., 1999). However, not all PS1 mutations lead to neuronal loss in a similar way. Hashimoto *et al.* examined 27 different FAD linked mutants of PS1, and found that N- and C-terminal PS1 mutants can generate distinct neurotoxic signals(Hashimoto et al., 2004). Thus, the PS1 mutants whose mutations are located in the N-terminal, cause NOS inhibitor-sensitive neurotoxicity. On the other hand, the PS1 mutants whose mutations are located in the C-terminal, cause NADPH oxidase inhibitor-sensitive neurotoxicity. This suggests that different mutations in PS1 might lead to neuronal cell death through different toxic mechanisms. We were able to show that unlike wild type PS1 both N and C-terminus mutations of PS1 were not able to stimulate the transcriptional activation ability of CBP. Moreover, PS1 C-terminus mutations showed a decrease in CBP transcriptional activity compared to control vector or PS1 N-terminus mutations.

Evidently, all our experiments investigating the effect of PS1 on CBP use Gal4/CBP fusion proteins, and this approach has been criticized, because of the artificial nature of the constructs used(Cardinaux et al., 2000). Here, the results have been independently confirmed using confocal immunofluorescence, which showed the different effect of wild type and mutated PS1 on the cellular localization of CBP. These findings clearly show that CBP, which in its active form is found in the nucleus, is activated by the wild type PS1 and not by mutant PS1.

A variety of recent data suggests that CBP is a target for specific signalling pathways. Interestingly, the C terminus of CBP, which mediates the strongest response to WT PS1, was also identified to be involved in its response to phenylephrine(Gusterson et al., 2002). Moreover, previous work has demonstrated that NGF was able to enhance CBP-Gal 4 activity via the p42/p44 MAPK pathway(Liu et al., 1998). The work presented here extends these studies to show wild type PS1 activates CBP and that this activity is dependent upon its ability to activate the PI 3-kinase, p38 MAP kinase and p42/p44 MAPK pathways. In addition, the data presented here suggests that different regions of CBP, primarily the C terminus, are activated by WT-PS1, but not by its mutant. This is of interest since recent studies have revealed that the C-terminal activation domain of CBP interacts with components of the basal transcriptional machinery such as TFIIB72 and the RNA polymerase holoenzyme complex as well as the transcriptional co-activator p/CIP(Kee et al., 1996; Torchia et al., 1997). Moreover, the increased activity of the CBP region containing amino acids 721-1679 induced by wild type PS1 was of particular interest since it contains the CBP acetyltransferase domain(Martinez-Balbas et al., 1998).

As mentioned in section 1.9, Saura *et al* have shown a reduction in CBP levels and CRE-dependent gene expression in their PS cDKO (Saura et al., 2004). However, they did not show if the reduction of CRE-dependent gene expression is a result of a decrease in CBP activity as well as a decrease in CBP levels. Here we used an *in vitro* approach to confirm the results of Saura *et al* by showing a decrease in CBP levels when endogenous PS1 was knocked down. Moreover, we were able to show, by using luciferase assay and Gal4 western blot, that when

PS1 is knocked down there is a decrease in CBP levels and there is a decrease in the transcriptional activation ability of CBP.

Importantly, in all of the experiments above we were able to show that the Gal-CBP luciferase results are not explained by changes in Gal4-CBP levels. Here the effects on the Gal4 promoter are due to by changes in CBP activity, enhancing its ability to activate transcription.

Chapter 4

4 THE EFFECTS OF PS1 ON P300

4.1 Introduction

As mentioned in section 1.8, it has been shown that CBP, but not p300, is involved in AD development (Teo et al., 2005). This result is perplexing since, as mention in section 1.6, CBP and p300 have a high degree of homology and similar patterns of expression. Moreover, p300 and CBP were shown to response similarly, but not identically, to different cell stimuli. Thus, we would expect that p300 would show similar level of involvement in AD as CBP showed in the last chapter. We therefore decided to examine the potential part of p300 in AD. In this chapter, the role of p300 in AD is tested by studying p300 activity and the effects of manipulating its expression in cells containing wild type or mutant forms of the proteins that are mutated in the FAD.

4.2 The effect of WT/mutant PS1 on the transcriptional activity of p300

To investigate the potential role of p300 in Alzheimer's disease we co-transfected Gal-p300 constructs with a plasmid containing a test promoter with DNA binding sites for Gal4 linked to the luciferase reporter gene. This allows the effect of any stimulus on the ability of p300 to activate transcription to be assessed without any complications arising from it being recruited to the DNA via interaction with a DNA-bound transcription factor that may itself be modulated by the stimulus. We have previously used this system to demonstrate that NGF stimulates p300 activity in neuronal cells(Liu et al., 1998) and to demonstrate the effect of PS1 on CBP transcriptional activity in the previous chapter .

The Gal-p300 construct was therefore transfected into F11/EcR line together with the reporter luciferase construct, and the cells were either left untreated or transfected with wild type or mutated PS1and EcD used to induce PS1expression. In these experiments, a significant enhancement of p300 driven promoter activity was observed in the cells transfected with constructs containing wild type Presenilin 1, compared to control where Gal-p300 was transfected with an empty vector (Fig 4.1). Remarkably, the PS1M146L mutant form produced virtually no enhancement in promoter activity, showing similar activity levels to the untreated cells. These results demonstrate that the PS1M146L mutant form, unlike wild type Presenilin 1, has no ability to activate p300 (Fig 4.1). In addition, virtually no enhancement in p300 activity was observed, in the absence of PS1transfection, when cells were treated with EcD, showing that EcD itself has no effect on p300 activity (Fig 4.2). These experiments were routinely done with the addition of EcD to induce PS1expression. Moreover, no enhancement in p300 activity was observed, in the absence of EcD, when the cells were transfected with wild type PS1 (Fig 4.2).

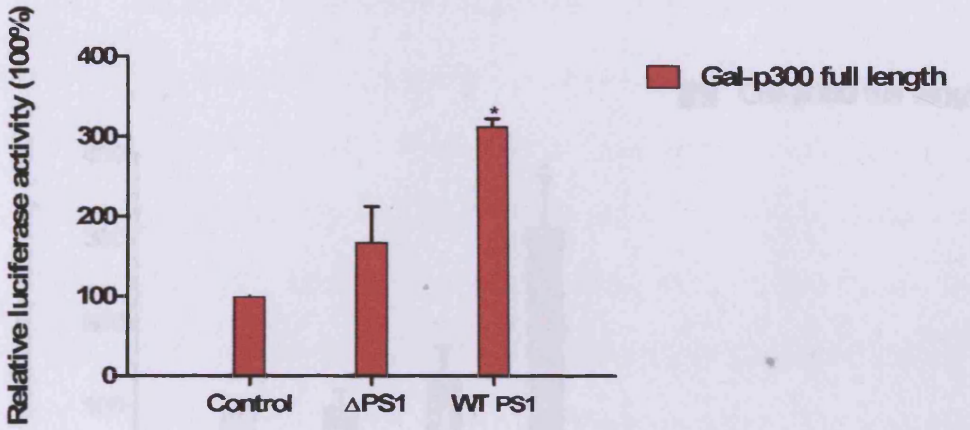

Figure 4.1 Effect of with wild type or mutated PS1on the transcriptional activity of p300 in F11 cells.

Luciferase assay results of co-transfecting F11 cells with 2 µg of the Gal4-E1B TATA-luciferase, 2 µg of the Gal-p300 (full length)-in the presence or absence of with wild type or mutated PS1(1 µg). Values are expressed relative to the level of luciferase in control, neural cells transfected with Gal-luciferase and Gal-p300 (set at 100%) and are the means of three independent transfection experiments whose standard error is indicated by the *bars*. *, $p < 0.05$ *versus* control.

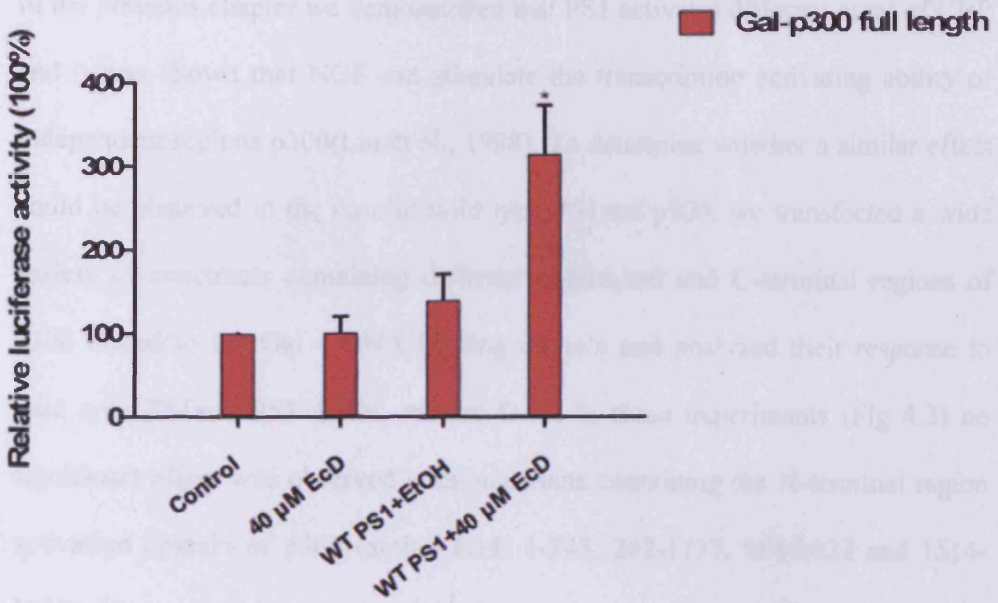

Figure 4.2 Effect of Ecd and wild type PS1 on the transcriptional activity of p300 in F11 cells.

Luciferase assay results of co-transfecting F11 cells with 2 µg of the Gal4-E1B TATA-luciferase, 2 µg of the Gal-p300 (full length)-in the presence or absence of with wild type PS1 (1 µg) -in the presence or absence of EcD (40 µM). Values are expressed relative to the level of luciferase in control, neural cells transfected with Gal-luciferase and Gal-p300 (set at 100%) and are the means of three independent transfection experiments whose standard error is indicated by the bars. *, $p < 0.05$ versus control.

4.3 Regions of p300 involved in activation by WT PS1

In the previous chapter we demonstrated that PS1 activates different areas of CBP and it was shown that NGF can stimulate the transcription activating ability of independent regions p300(Liu et al., 1998). To determine whether a similar effect could be observed in the case of wild type PS1and p300, we transfected a wide variety of constructs containing different N-terminal and C-terminal regions of p300 linked to the Gal 4 DNA binding domain and analyzed their response to wild type PS1and PS1M146L mutant form. In these experiments (Fig 4.3) no significant effect was observed with constructs containing the N-terminal region activation domain of p300 (amino acids 1-743, 242-1737, 964-1922 and 1514-1922). However, a decreased activity in response to wild type PS1and its mutant was shown with the construct containing the region of p300 from amino acids 1737-2414 (Fig 4.3). In addition, decreased activity in response to wild type PS1and a small non-statistically significant effect of mutant PS1 was observed in the construct containing amino acids 1945- 2414.

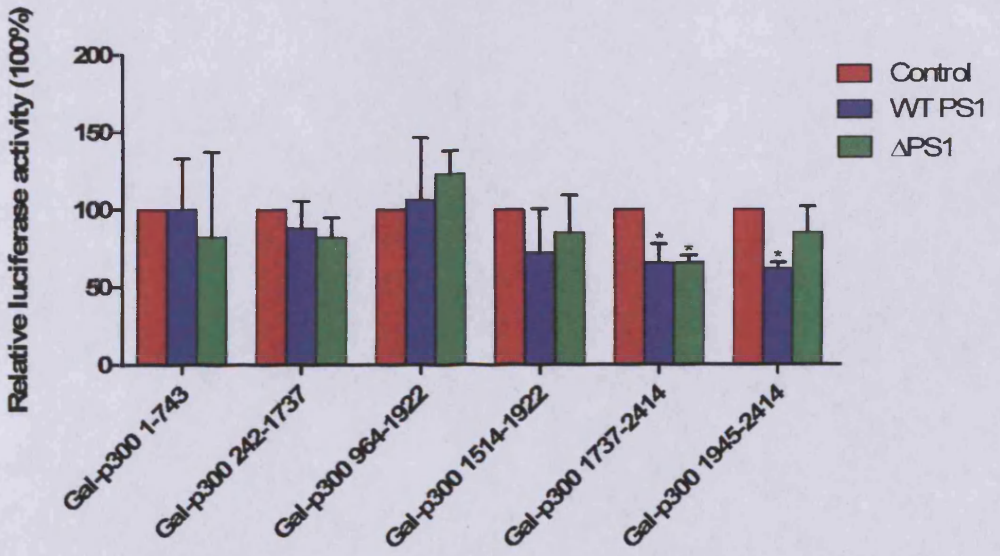

Figure 4.3 Effect of wild type PS1on the transcriptional activity of different N-terminal and C-terminal regions of p300 in neural cell.

Luciferase assay results of co-transfecting F11 cells with 2 μg of the Gal4-E1B TATA-luciferase together with 2 μg of the p300 C-terminal regions (amino acids 1514-1922, 1737-2414 or 1945-2414) or p300 N-terminal regions (amino 1-743, 242-1737 or 964-1922) linked to Gal4 in the presence of wild type PS1(1 μg). In each case values are expressed relative to the level of luciferase in untreated F11 cells transfected with each construct (set at 100%) and are the means of three independent transfection experiments whose standard error is indicated by the bars. *, $p < 0.05$ versus the same construct without wild type Presenilin 1.

4.4 Discussion

Similar to CBP, p300 has been shown to be a co-activator for a number of transcription factors (see section 1.6). Gene expression has been shown to play a role in the consolidation of long-term forms of synaptic plasticity and memory(Silva et al., 1998). In the case of FAD, initial study showed that PS1 L286V mutant increased TCF/beta-catenin/CBP-mediated transcription. On the other hand, PS1 L286V mutant failed to activate TCF/beta-catenin/p300-mediated transcription, which lead to a decreas in neuronal differentiation (Teo et al., 2005). However, regulation of p300 activity in response to wild type Presenilin 1 and its M146L mutant has not previously been reported.

The data presented here add a new aspect to the mechanism by which the mutant of PS1 is involved in AD. Thus, wild type Presenilin 1, but not its M146L mutant form, was found to stimulate the transcriptional activation ability of p300. In addition, we showed that wild type Presenilin 1 and its M146L mutant form failed to increase the transcriptional activity of the N-terminal domain of p300 and decreased the transcriptional activation ability of the p300 C-terminal domain. This is surprising since the C-terminal domain of p300 had been shown to interact with the RNA polymerase holoenzyme complex as well as the transcriptional co-activator p/CIP(Torchia et al., 1997). Moreover, p300 C-terminal region has been shown to be activated by phenylephrine and the p300 C-terminal homolog and region in CBP was demonstrated to be activated by WT PS1, showing that specific regions of CBP or p300 are involved in those effects. However, despite their high homology, the same signalling pathways were shown to target different regions of CBP and p300 to enhance their transcriptional activating ability

(Gusterson et al., 2002). It is seems that the whole of p300 molecule is needed to get the activation effect of wt PS1.

The stimulation of the transcriptional activation ability of p300 by wild type Presenilin 1, but not by its M146L mutant form, supports a mechanism in which mutation of Presenilin 1 reduces p300 activity rather than enhancing it (Teo et al., 2005). In addition, although that the precise domains of p300 which are activated as a response to wild type PS1 remain to be identified, it is already clear that the transcriptional activating ability of this critical co-activator is stimulated by wild type PS1 in neuronal cells. In addition, the M146L Presenilin 1 mutant shows partial loss of function and may lead to reduced gene expression, which will cause memory loss and neurodegeneration in AD.

The original goal of the work described in this chapter was to examine if the same general effects observed for CBP in chapter 3 also apply to p300. Moreover, we wanted to explore the possibility that p300 is involved in AD, since this was never shown before (Teo et al., 2005). So far, the results we obtained were very similar to those obtained for CBP. Therefore this work has not yet been extended further. It would be interesting in the future to perform confocal immunofluorescence experiments that would determine if wild type PS1/PS1 mutants have similar effects on p300 cellular localization, to those observed in the case of CBP. In addition, it would be of value to test a wider range of PS1 mutants for their effect on p300 transcriptional activity as shown in chapter 3 for CBP.

Chapter 5

5 INVOLVMENT OF CBP HISTONE ACETYLTRANSFERASE ACTIVITY IN ALZHEIMER'S DISEASE

5.1 Introduction

As mentioned in section 1.2.1, the acetylation of histones creates a more open chromatin structure, which makes the DNA more accessible to the transcriptional machinery and regulates the transcription of numerous genes. Furthermore, by acetylation of histones, CBP induces chromatin changes that results in the loss of transcriptional repression (see section 1.6). As shown in section 3.2 and by Saura *et al.* (Saura et al., 2004), CBP is involved in the development of AD. However, the mechanism of CBP involvement in the disease is unclear. The increased activity of the CBP region containing amino acids 721-1679 induced by wild type PS1, which was demonstrated in section 3.5, was of particular interest since it contains the CBP acetyltransferase domain(Martinez-Balbas et al., 1998). We therefore decided to determine whether the histone acetyltransferase activity of CBP has any role in the stimulatory effect of WT-PS1. Here we will test a mechanism by which WT PS1 stimulates CBP to induce gene transcription and in particular relate it to its known HAT activity.

5.2 The effect of WT/mutant PS1 on c-fos luciferase expression

To further investigate the potential role CBP HAT domain in Alzheimer's disease, we wished to test the role of CBP in the activation of a natural promoter by WT PS1. To do this we used the promoter of the c-fos gene in a reporter luciferase construct. It has been shown that c-fos is one of the genes induced in the brain by

CBP and is involved in memory formation and consolidation(Tischmeyer and Grimm, 1999). It has been demonstrated that the histone acetyltransferase activity of CBP is required for normal c-fos promoter activation and that c-fos levels are significantly decreased in CBP [HAT−] mice(Korzus et al., 2004). As a result we decided to examine the effect of PS1 on the c-fos promoter.

The c-fos luciferase construct was therefore transfected into the F11/EcR line and the cells were either left untreated or transfected with wild-type or mutated Presenilin 1. EcD was used to induce Presenilin 1 expression. c-fos luciferase expression was activated by the wild type PS1 but not by its M146L mutant (Fig 5.1A). In addition the activation of the c-fos promoter by WT PS1 was abolished by co-transfection of a construct expressing full-length CBP in an antisense orientation (Fig 5.1B), indicating that CBP was involved in this effect. Hence, the role of CBP in the PS1 response can be demonstrated on a natural promoter by inhibiting endogenous CBP providing further evidence of the importance of the HAT region in this activation. This shows that WT PS1 affects c-fos expression and it is CBP dependent. In addition, virtually no enhancement in c-fos luciferase expression was observed, in the absence of Presenilin 1 transfection, when cells were treated with EcD, showing that EcD itself has no effect on c-fos promoter activity (Fig 5.2A). These experiments were routinely done with the addition of EcD to induce Presenilin 1 expression. No enhancement in c-fos luciferase expression was observed, in the absence of EcD, when the cells were transfected with wild type Presenilin 1 (Fig 5.2A).

Moreover, evidence to show that CBP antisense eliminates CBP endogenous expression was provided using western blot analysis. Experiments were

preformed in which F11 cells were transfected with antisense CBP or an empty expression vector. The result of this experiment showed a dramatic decrease in levels of endogenous CBP (fig 5.3), confirming that the cells transfected with antisense CBP show greatly reduced CBP expression.

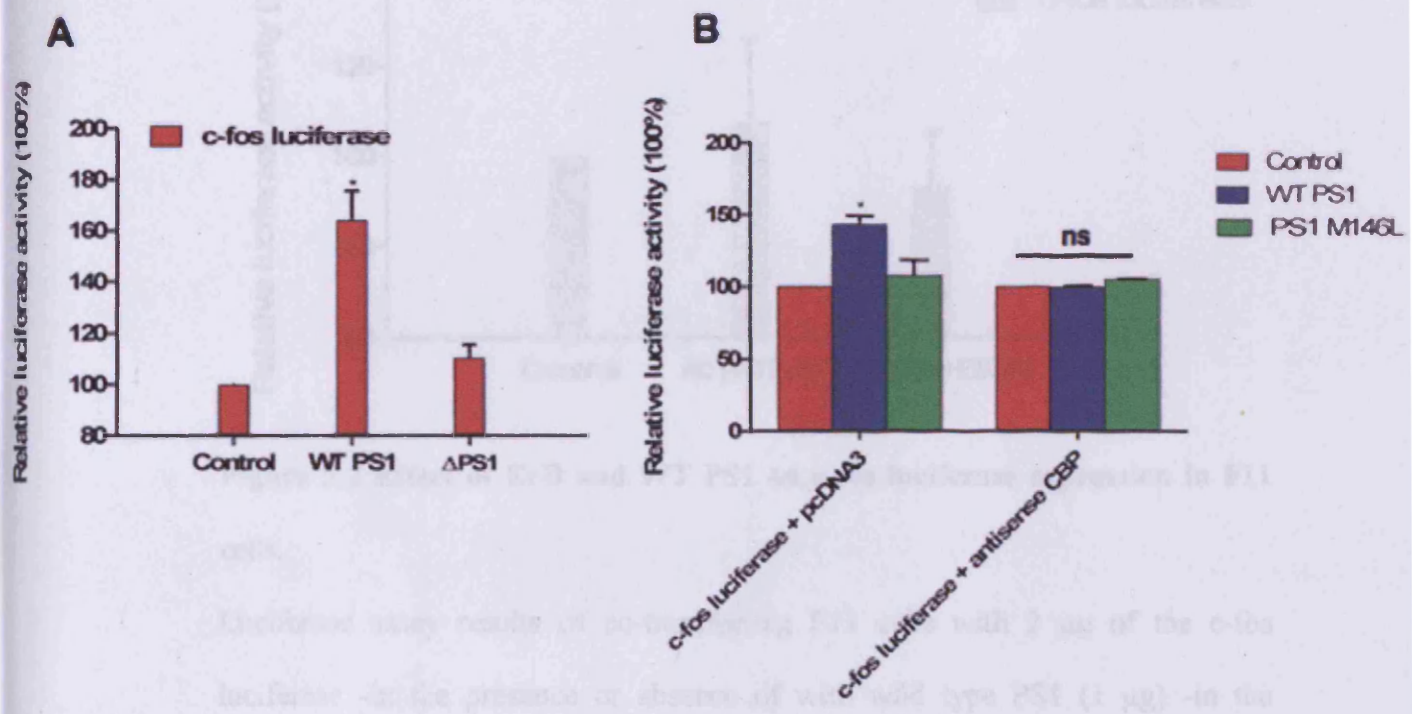

Figure 5.1 Effect of the wild type and mutant PS1 on c-fos luciferase expression.

A) Luciferase assay results of co-transfecting F11 cells with 2 μg of the c-fos luciferase together with 1 μg of wild-type 1 or mutant presenilin. Values are expressed relative to the level of luciferase in control (neural cells transfected with c-fos luciferase and 1 μg of empty vector) set at 100%. Values are the means of 3 independent transfection experiments whose s.e. is indicated by the bars. * $p<0.05$ vs control. B) Luciferase assay results of co-transfecting F11 cells with 2 μg of the c-fos luciferase together with 1 μg of wild-type 1 or mutant presenilin and with either 2.5 μg of empty expression vector or 2.5 μg of CBP antisense plasmid. Values are expressed relative to the level of luciferase in control (neural cells transfected with c-fos luciferase and transfected empty vector) set at 100%. No significant difference was found.

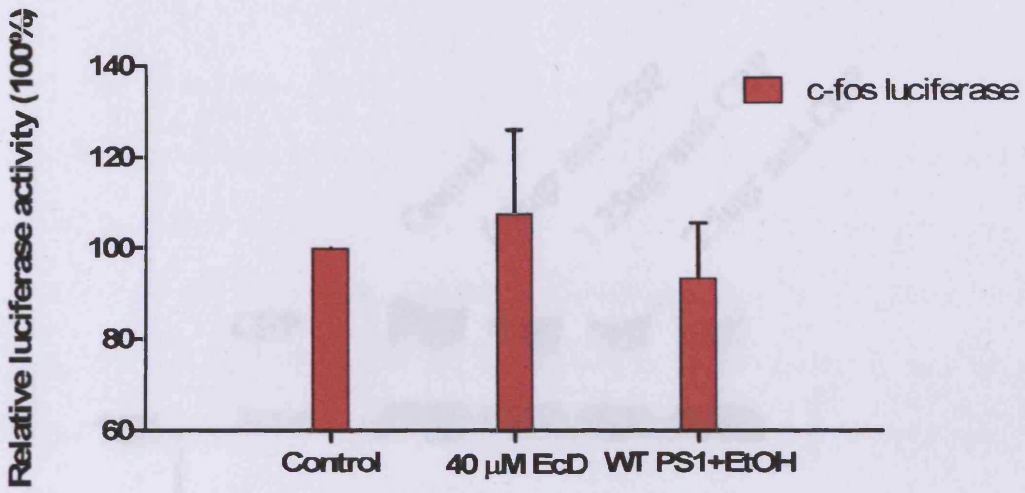

Figure 5.2 Effect of EcD and WT PS1 on c-fos luciferase expression in F11 cells.

Luciferase assay results of co-transfecting F11 cells with 2 µg of the c-fos luciferase -in the presence or absence of with wild type PS1 (1 µg) -in the presence or absence of EcD (40 µM). Values are expressed relative to the level of luciferase in control, neural cells transfected with Gal-luciferase and Gal-CBP (set at 100%) and are the means of three independent transfection experiments whose standard error is indicated by the bars. $p > 0.05$ versus control.

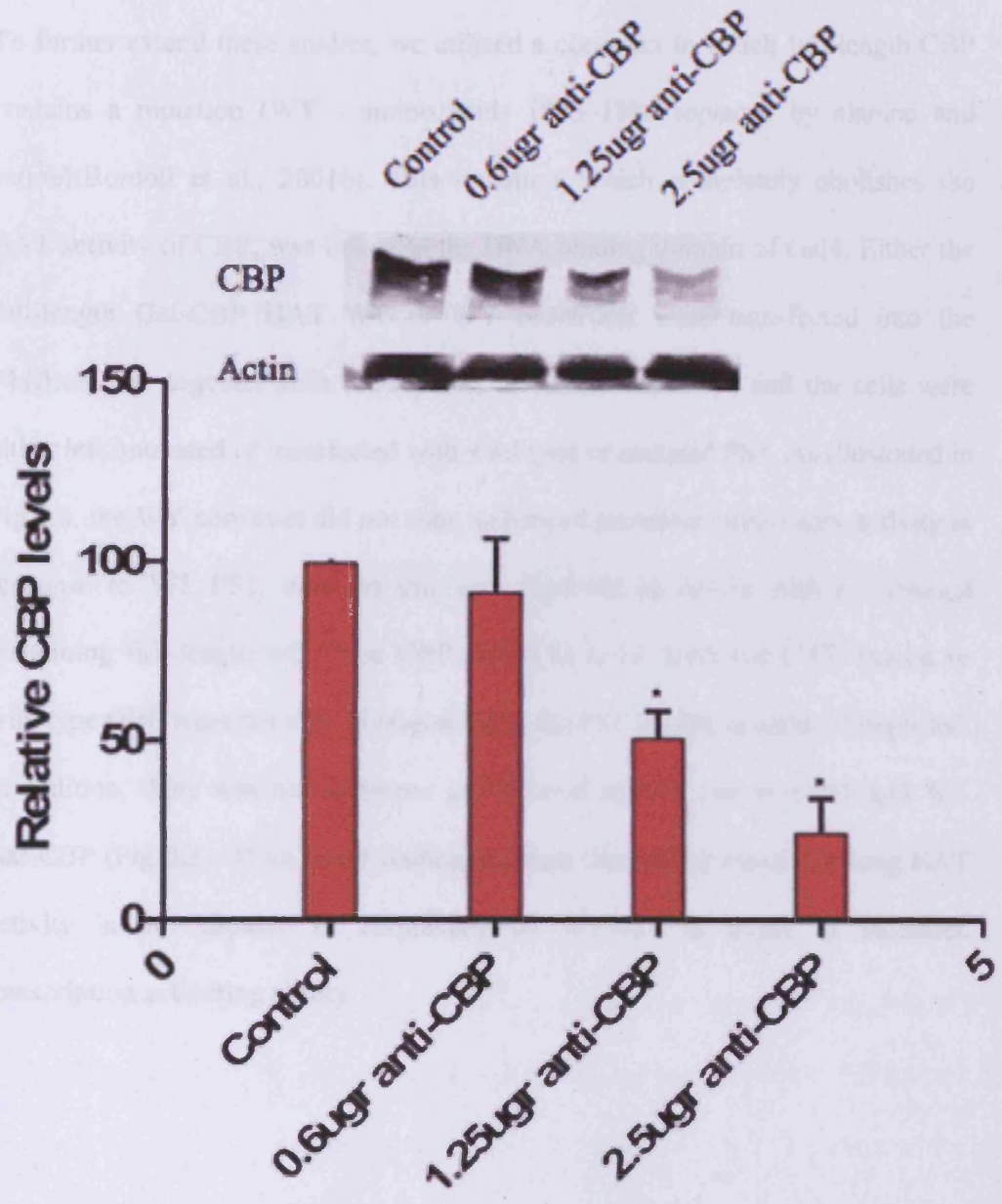

Figure 5.3 Effect of CBP antisense on endogenous CBP levels

Expression if CBP in F11 cells either with an empty vector (2.5 μgr) or with different concentrations of CBP antisense construct. Values are means of three independent transfection experiments whose standard error is indicated by the bars. $p > 0.05$ versus control. Results were normalized against the control. *, $p < 0.05$ versus control

5.3 CBP, but not its HAT mutant, is stimulated by WT PS1

To further extend these studies, we utilized a construct in which full-length CBP contains a mutation (WY - amino acids 1503–1504 replaced by alanine and serine)(Bordoli et al., 2001b). This mutation, which completely abolishes the HAT activity of CBP, was linked to the DNA binding domain of Gal4. Either the full-length Gal-CBP HAT WT or WY constructs were transfected into the F11/EcR line together with the reporter luciferase construct, and the cells were either left untreated or transfected with wild-type or mutated PS1. As illustrated in Fig 5.3, the WY construct did not show enhanced promoter stimulatory activity in response to WT PS1, whereas this was observed as before with a construct containing full-length wild type CBP linked to Gal4. Both the HAT mutant or wild type CBP were not able to responded to the PS1 M146L mutant, as expected. In addition, there was no difference in the basal activity between WT and WY Gal-CBP (Fig 5.3). Thus, these findings suggest that a CBP mutant lacking HAT activity is not capable of responding to WT-PS1 in terms of increased transcription activating ability.

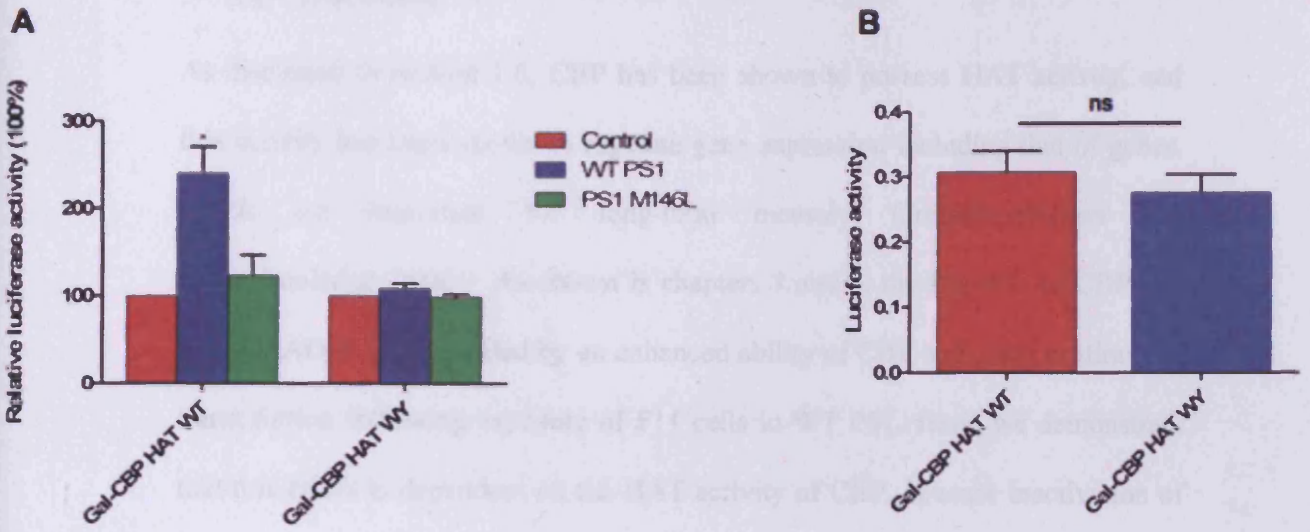

Figure 5.4 Relative luciferase Gal4 promoter activity of either WT-CBP or its mutant lacking HAT activity (WY-CBP).

A) Luciferase assay results of co-transfecting F11 cells with 2 μg of the Gal4-E1B TATA-luciferase together with 2 μg of the luciferase gene and constructs containing either CBP WT or CBP WY linked to the DNA binding domain of Gal4 in the presence of wild-type or mutant presenilin 1 (1 μg). Values are expressed relative to the level of luciferase in control (neural cells transfected with Gal-luciferase, Gal-CBP and 1 μg of empty vector) set at 100%. Values are the means of 3 independent transfection experiments whose s.e. is indicated by the bars. * $p<0.05$ vs control. B) Basal luciferase activity in F11 cells co-transfected with 2μg of Gal4 DNA binding sites upstream of the luciferase gene and constructs containing either CBP WT or CBP WY linked to the DNA binding domain of Gal4. Values are expressed as the light intensity. No significant difference was found.

5.4 Discussion

As discussed in section 1.6, CBP has been shown to possess HAT activity, and this activity has been shown to regulate gene expression including that of genes, which are important for long-term memory formation(Hallam and Bourtchouladze, 2006). As shown is chapters 3 and 4, the key role of CBP and p300 in AD is accompanied by an enhanced ability of CBP and p300 to stimulate transcription following exposure of F11 cells to WT PS1. Here, we demonstrate that this effect is dependent on the HAT activity of CBP, because inactivation of such activity within a construct containing CBP linked to the Gal-4 DNA binding domain abolishes the enhanced activity normally observed in response to WT PS1.

To further extend these studies, we tested the role of CBP in the activation of a natural promoter by WT PS1. To do this we used the promoter of the c-fos gene, which is involved in memory formation and is depended on CBP HAT activity(Tischmeyer and Grimm, 1999). We showed that c-fos luciferase expression was activated by the wild type WT PS1. Remarkably, the Presenilin 1 M146L mutant form produced virtually no enhancement in promoter activity. Moreover, the activation of this promoter by WT PS1 was abolished by co-transfection of a construct expressing full-length CBP in an antisense orientation indicating that it was able to prevent a well characterized effect of CBP. Hence, the role of CBP in the WT PS1 response can be demonstrated on a natural promoter by inhibiting endogenous CBP providing further evidence that it is not dependent on the use of Gal4-CBP constructs.

Furthermore, c-fos gene induction by CBP is depended by CBP HAT domain (Korzus et al., 2004). This suggests that the c-fos decreased in promoter activity in cells transfected with Presenilin 1 M146L mutant might be as a result of a decrease in CBP HAT activity. Therefore, the HAT activity of CBP appears to be essential for enhancing transcriptional activity ability in the presence of WT PS1.

The data presented here shows for the first time that CBP HAT region plays a key role in the response to WT PS1 stimulus and suggests that PS1 mutant form is not able to stimulate CBP HAT activity.

Chapter 6

6 BENEFICIAL EFFECT OF HISTONE DEACETYLASES INHIBITOR TSA IN A MOUSE MODEL OF ALZHEIMER'S DISEASE

6.1 Introduction

The data presented in chapters 3 and 5 suggests that enhanced histone acetylation might reestablish normal gene transcription in AD. This histone acetylation enhancement could be achieved in three main ways:

1. Overexpression of CBP
2. Specific enhancement of CBP HAT activity
3. Inhibition of histone deacetylation

Although all of the above approaches lead to histone acetylation enhancement, overexpression of CBP in an *in vivo* model involves some technical challenges. In addition, drugs for specific enhancement of CBP HAT activity are commercially unavailable. Therefore, it was decided to explore HDAC inhibitors to produce histone acetylation enhancement

As mentioned in section 1.2.1, HDACs remove an acetyl group from histones, thus restricting access of the transcriptional machinery to the DNA. HDAC inhibitors have been shown to enhance LTP and memory and learning in rats (Levenson et al., 2004). Moreover, in a transgenic mouse model of HD, administration of HDAC inhibitors improved brain morphology, survival, body weight and motor performance (Ferrante et al., 2003). Also, memory and LTP

deficits of $CBP^{+/-}$ mice were reversed by the administration of HDAC inhibitor, suberoylanilide hydroxamic acid (SAHA), supporting the potential use of this class of drugs as therapeutic agents against mental retardation in RTS(Alarcon et al., 2004). Interestingly, HDAC inhibitors, such as SAHA and TSA, are currently being used in clinical trials for the treatment of some forms of cancer, based on their ability to suppress tumor cell proliferation by activating the transcription of genes involved in cell-cycle arrest, differentiation and/or apoptosis (Marks et al., 2004). Given the role of the CBP HAT domain in AD and the positive effects of HDAC inhibitors in reducing neurodegeneration and improving survival and impaired behaviour in animal models of HD and RTS, this drug might also be effective in therapeutic approaches against AD (Fig 6.1).

However, F11 cell line test conditions may not correspond to the conditions inside of the organism. Therefore, it was decided to extend our *in vitro* results with *in vivo* research. Diverse opportunities to examine the molecular basis of AD and intervene with the mechanisms causing the disorder are provided by transgenic mouse models overexpressing mutant forms of APP, PS1 and PS2(Wong et al., 2002). One of the most well-known models is the transgenic mouse expressing the familial Swedish mutation K670N:M671L in APP. This mouse shows increase of brain Aβ levels by 6-8 months of age followed by development of neuritic plaques in the neocortex and hippocampus(Frautschy et al., 1998; Hsiao et al., 1996; Irizarry et al., 1997) and impairment of the cognitive function by 9-10 months(Chapman et al., 1999; Holcomb et al., 1998). Interestingly, the AD-like pathology is even more accentuated in mice obtained by crossing APP animals with the line 6.2 of PS1 (M146L) animals, which over express the FAD-linked

PS1 M146L mutation. Consistent with studies in which a mutated APP gene is co-expressed with a mutated PS1 gene (Borchelt et al., 1997), the APP/PS1 (line 6.2) mouse starts developing plaques at about 2 months(McGowan et al., 1999). Moreover, they have reduction in LTP by 3 months of age together with contextual fear memory impairment and spatial working memory. These findings suggest that AD-like dysfunctions might occur at earlier stages in double transgenic APP/PS1 mice compared with transgenic models with a single APP or PS1 mutation. Because of the faster appearance of their phenotype, double transgenic mice are currently used by several laboratories for studies on the mechanisms of AD as well as for the development of a therapy to prevent and/or treat the disease. Consequently, it was decided to use an APP/PS1 AD mice model for our experiments.

Figure 6.1 HDAC inhibition.

6.2 Effect of TSA on synaptic function in hippocampal slices of APP/PS1 mice

In the first series of experiments it was tested whether an inhibitor of histone de-acetylation can re-establish normal synaptic function in the APP/PS1 mouse. Synaptic dysfunction is thought to be one of the first alterations occurring in AD. Thus, a series of experiments were performed to tested whether a brief application of the HDAC inhibitor, TSA, was capable of rescuing the defect in LTP shown by brain hippocampal slices from 3 month-old APP/PS1 mice. At this age the APP/PS1 mice start showing synaptic plasticity impairment (Trinchese et al., 2004). Hippocampal slices were perfused with TSA (1.65μM) for 30min before inducing late-phase LTP (L-LTP) through tetanic stimulation of the Schaeffer collateral pathway.

It was shown that potentiation in TSA treated APP/PS1 slices were far greater than in vehicle-treated APP/PS1 slices (Fig 6.2A). Two-way ANOVA revealed a significant difference between the two APP/PS1 groups and a WT comparisons showed that the groups were significantly different at each time point after the tetanus. On the other hand, TSA did not change the amplitude of LTP in hippocampal slices of WT mice compared to WT slices treated with vehicle alone (Fig. 6.2B). Moreover, TSA had no effect in the basal synaptic responses in slices from APP/PS1 mice or WT littermates either during TSA application or 120 min after the end of the application in experiments in which no tetanic stimulation was applied (n=3; Fig 6.2). Thus, this shows that inhibitors of histone de-acetylation are able to re-establish normal synaptic function in the APP/PS1 mouse.

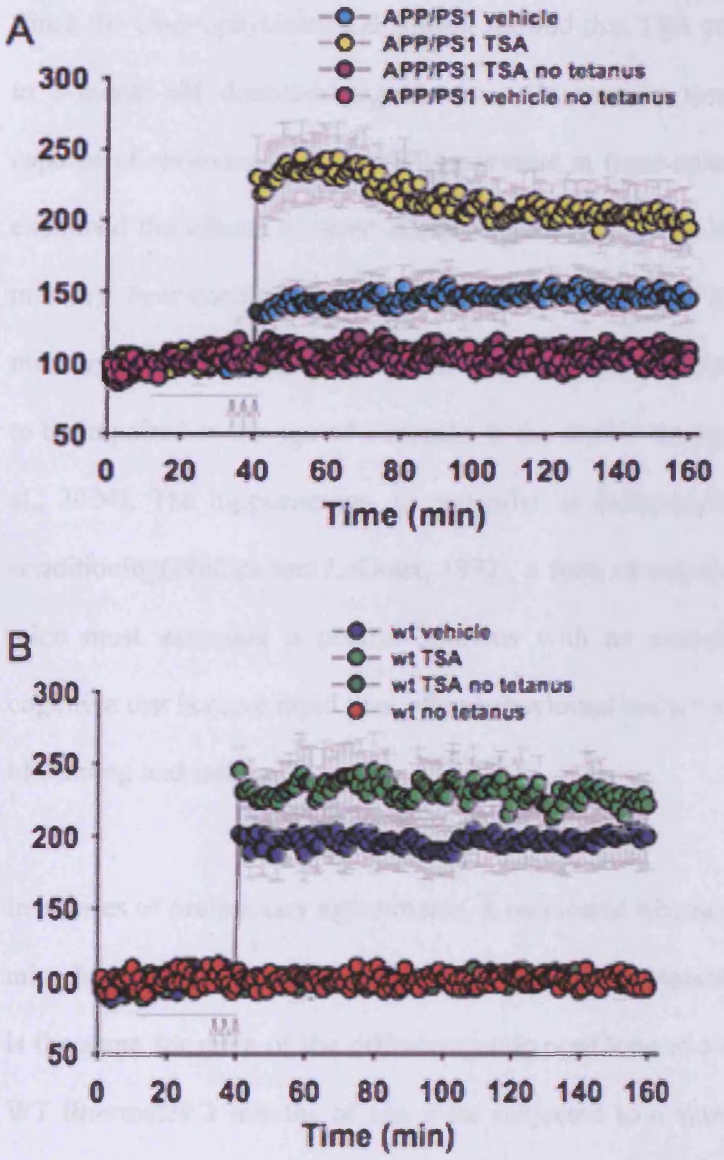

Figure 6.2 TSA reverses CA1-LTP impairment in slices from APP/PS1 mice.

(A) Summary of the graph showing that 30 min of HDAC treatment abrogates LTP impairment in APP/PS1 mice without affecting the baseline transmission (n=5, P<0.005). (B) Summary graph showing that 30min of treatment with TSA does not effect LTP and baseline transmission in WT mice (n=5, P>0.05). These experiments were interleaved with those of WT mice. The horizontal bar represents TSA application. The 3 arrows correspond to the theta-burst.

6.3 Effect of TSA on the cognitive function of APP/PS1 mice

Since the electrophysiological studies showed that TSA ameliorated LTP deficits in 3-month-old double-transgenic mice, Itwas then tested whether TSA was capable of reversing behavioural impairment in these animals. Our initial studies examined the effects of acute administration of TSA on hippocampus-dependent memory. Fear-conditioning learning was used as a test of hippocampus-dependent memory. This test involves different forms of explicit learning and has been found to be impaired at the age of 3 months in the double-transgenic mice(Trinchese et al., 2004). The hippocampus, in particular, is indispensable for contextual fear conditioning(Phillips and LeDoux, 1992), a form of associative learning in which mice must associate a neutral stimulus with an aversive one. This type of cognitive test is more rapid than other behavioural tasks that require multiple days of training and testing.

In a series of preliminary experiments, it was tested whether the APP/PS1 and WT mice had different perceptions of an electric shock. Notably, the shock threshold is the same for mice of the different genotypes(Gong et al., 2004). APP/PS1 and WT littermates 3 months of age were subjected to a standard fear-conditioning paradigm(Bourtchuladze et al., 1994). 2 hours after injection of TSA (2 μgr/gr body weight; i.p), the animals were placed in a novel context (fear-conditioning box) and were exposed to a mild foot shock (training phase of the fear conditioning). Conditioning was assessed 24 hours later by measurement of "freezing" behaviour — the absence of all movement except for that necessitated by breathing — in response to the context (contextual conditioning).

During the training phase, no difference in the freezing of APP/PS1 mice treated with TSA or with vehicle compared with that of TSA- or vehicle-treated WT littermates was found (Fig 6.3A). Two-way ANOVA followed by the Bonferroni test revealed no significant difference between the 4 groups (Fig 6.3A) [$P > 0.05$].

Twenty-four hours later we found a decrease in the freezing time of vehicle-treated APP/PS1 mice compared with that of vehicle-treated WT littermates in contextual conditioning was demonstrated (vehicle-treated APP/PS1 mice demonstrated about 46% that of vehicle-treated WT mice; 14.88 ± 3.22% in APP/PS1, n = 13, 8 females plus 2 males, versus 31.68 ± 5.31% in WT littermates, n = 13, 8 females plus 2 males; Fig 6.3B). However, the freezing time was increased in APP/PS1 mice after the injection of TSA (TSA-treated APP/PS1 mice demonstrated about 110% that of vehicle-treated WT mice: 35.04 ± 6.43%, n = 13, 8 females plus 2 males; Figure 6.3B).

The freezing responses of TSA-treated APP/PS1 mice were similar to those of vehicle-treated WT littermates. WT animals treated with TSA showed a nonsignificant increase in freezing (about 116% that of vehicle-treated WT mice: 36.95 ± 6.42%, n = 13, 8 females plus 2 males), probably because maximal levels of memory are already induced in vehicle-treated WT mice after the training session, as has been found both in Drosophila and in mice(Tully et al., 2003).

Two-way ANOVA followed by the Bonferroni test revealed a statistically significant difference between the 4 groups ($P < 0.01$). Post-hoc analysis showed a statistically significant difference between vehicle-treated APP/PS1 and vehicle-

treated WT littermates ($P < 0.05$) as well as between TSA-treated and vehicle-treated APP/PS1 mice ($P < 0.01$). Thus, this shows that inhibitors of histone de-acetylation are able to re-establish normal memory in the APP/PS1 mouse.

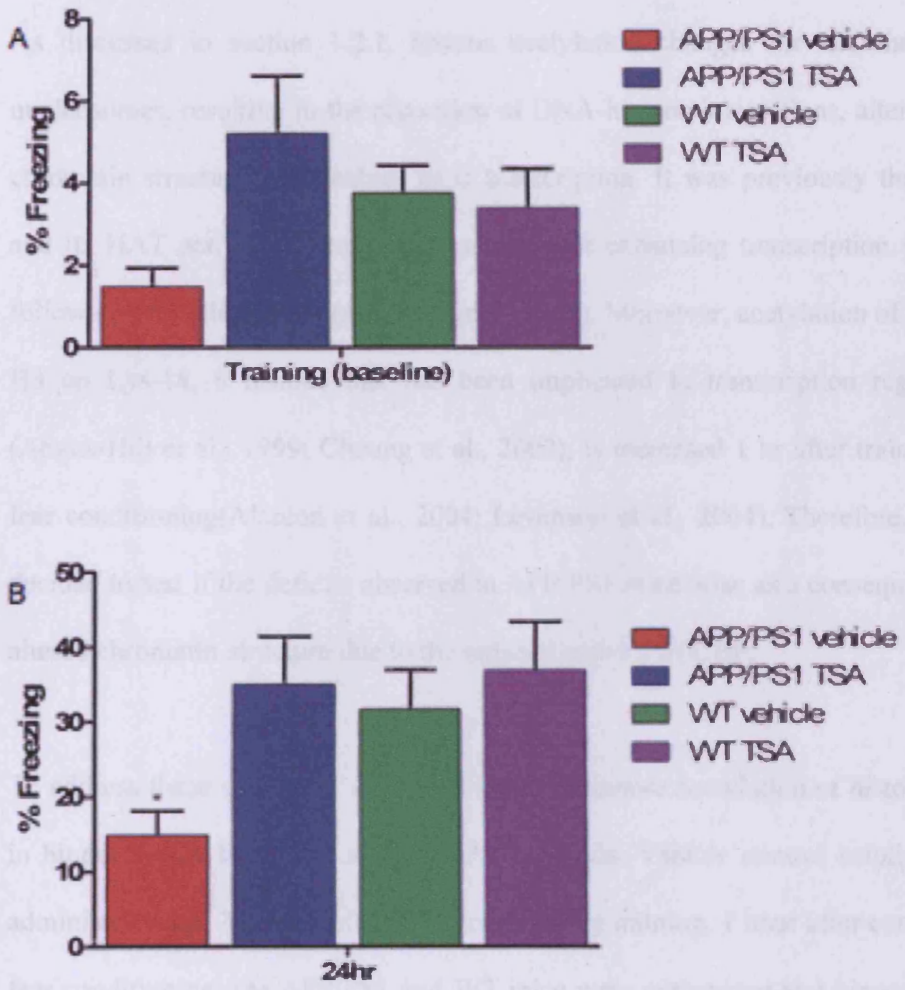

Figure 6.3 TSA injections improve contextual conditioning performance in 3-month-old APP/PS1 mice.

(A) Three-month-old APP/PS1 and WT littermates treated with TSA or vehicle 30 minutes prior to testing show no difference in immediate freezing in the training chamber ($P > 0.05$). (B) Three-month-old vehicle-treated APP/PS1 mice show reduced freezing responses compared with vehicle-treated WT littermates during contextual fear conditioning performed 24 hours after training. Injection of TSA the day before ameliorates the deficit in freezing responses in APP/PS1 mice. TSA has no effect on the freezing responses in WT mice ($P < 0.01$).

6.4 Effect of TSA on histone acetylation in APP/PS1 mice

As discussed in section 1.2.1, histone acetylation changes the net charge of nucleosomes, resulting in the relaxation of DNA-histone interactions, altering the chromatin structure that enables gene transcription. It was previously that CBP and its HAT activity appear to be essential for enhancing transcription *in vitro* following PS1 stimulation (see chapters 3 and 5). Moreover, acetylation of histone H3 on Lys-14, a residue that has been implicated in transcription regulation (Angus-Hill et al., 1999; Cheung et al., 2000), is increased 1 hr after training for fear conditioning(Alarcon et al., 2004; Levenson et al., 2004). Therefore, it was decided to test if the deficits observed in APP/PS1 mice arise as a consequence of altered chromatin structure due to the reduced activity of CBP.

To address these questions, it was decided to measure acetylation of histones H4 in hippocampus from WT and APP/PS1 animals. Vehicle control solution was administered i.p. 2 hours before fear conditioning training. 1 hour after contextual fear conditioning, the APP/PS1 and WT mice were euthanized and hippocampus was extracted. Western blot analysis of hippocampal extracts demonstrated that compare to WT miceAPP/PS1 mice showed an overall reduction of more than 50% in acetylated histone 4 levels (Figures 6.4). It was next asked whether HDAC inhibition might rescue the histone 4 acetylation levels defect observed in APP/PS1 mice. It was found that injection of TSA (2 μgr/gr body weight; i.p) 2 hours prior to contextual fear conditioning enhanced H4 acetylation of APP/PS1 mice (Fig 6.4). This demonstrates that AD is disease with an epigenetic motifes and that HDAC inhibitors are able to elevate the decreased levels of histone 4 in AD mouse.

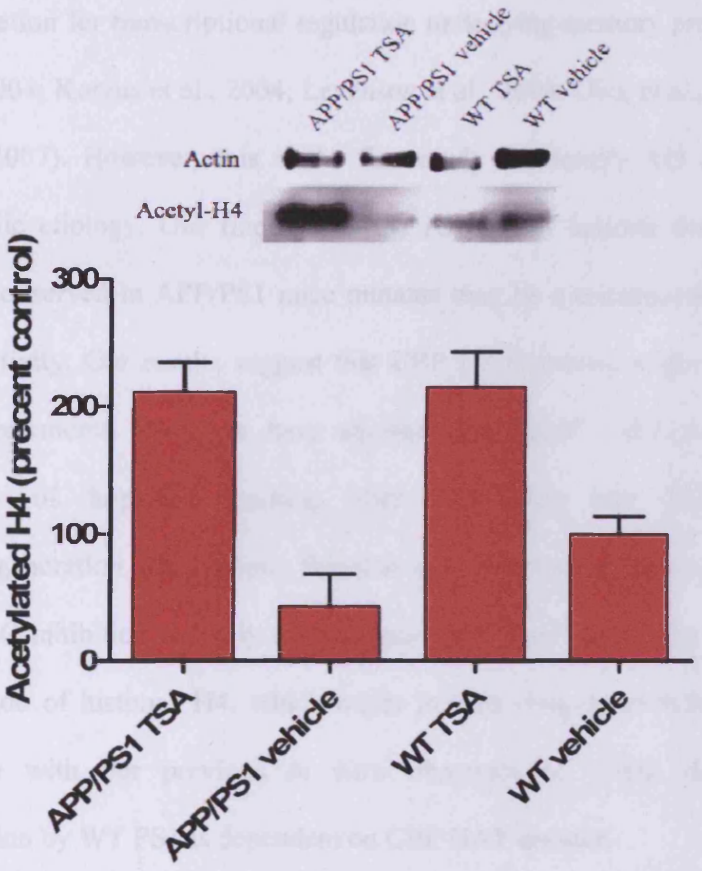

Figure 6.4 Rescue of histone H4 acetylation by TSA in 3-4 month old APP/PS1 mice.

Western blot using acetylated H4 antibody of protein extract from mice that were euthanized 1 hour after contextual fear conditioning. APP/PS1 and WT mice were treated with vehicle or TSA, 2 hours before training. This experiment was repeated three timed with similar results. Results were normalized against WT vehicle. *, $p < 0.05$ versus control.

6.5 Discussion

The use of HDAC inhibitors in rodents has demonstrated the role of chromatin modification for transcriptional regulation underlying memory processes(Alarcon et al., 2004; Korzus et al., 2004; Levenson et al., 2004; Oike et al., 1999b; Vecsey et al., 2007). However, this is the first study to identify AD as a disease of epigenetic etiology. Our findings on the AD model indicate that the cognitive deficits observed in APP/PS1 mice mutants may be a consequence of a reduced HAT activity. Our results suggest that CBP HAT activity might be involved in AD impairments. Here we have showed that HDAC inhibition enabled the recovery of impaired learning after AD mice had developed severe neurodegeneration and synaptic function loss. Our results suggest that the effect of HDAC inhibition is likely to be mediated, at least in part, by re-elevation in acetylation of histones H4, which might initiate synaptic growth. These results correlate with our previous *in vitro* observations, which show that CBP stimulation by WT PS1 is dependent on CBP HAT domain.

It is important to note that the effect of HDAC inhibitors on learning and memory could be a combination of modifications on chromatin and non-histone proteins, because it has been reported that some HDACs also target non-histone substrates(Yuan et al., 2005). Moreover, it is possible that different classes of HDACs are distinctly involved in synaptic plasticity. For example, it has been shown that HDAC5 is associated with downregulation of certain brain-derived neurotrophic factor (BDNF) transcripts in a social defeat paradigm, a phenomenon that can be reversed with HDAC inhibitor treatment(Tsankova et al., 2006).

It is unclear from these results if CBP or other HATs deregulations are the cause for H4 decreased of acetylation. However, it has been shown that the enhancement of hippocampus-dependent memory and hippocampal synaptic plasticity by HDAC inhibitors is mediated by the activation of key genes regulated by the CREB:CBP transcriptional complex(Vecsey et al., 2007). This suggests that HDAC inhibitors could be used to treat cognitive disorders that are associated with deregulated CBP activity or decreased CBP levels.

Interestingly, 'rewiring' of the brain and recovery of memory, using HDAC inhibitors, was recently reported in a neurodegenerative mice model(Fischer et al., 2007). Therefore, it is possible that HDAC inhibitors would be capable of re-establishing neural networks in AD brain model. If so, this suggests that using small molecules that target HDACs in AD patients could facilitate access to long-term memories.

The therapies for AD that are currently in use include augmentation of the cholinergic system by inhibition of acetylcholinesterases or, more recently, the use of NMDA antagonists that may act by blocking glutamate neurotoxicity. These agents have a limited efficacy and only the latter appears to have an even modest effect on the course of the disease. A major effort is underway to decrease the Aβ load in the brain either by the use of agents that block the secretases that process APP to Aβ or by the use of treatments, such as immunization with Aβ, that appear to augment the removal of Aβ from the brain(Schenk et al., 1999). Animal studies have demonstrated that the neuritic dystrophy that develops in animals that overproduce Aβ can be reversed by immunization (by Aβ antibodies)

that removes Aβ from the brain(Lombardo et al., 2003). Unfortunately, human trials of an Aβ vaccine had to be terminated because of encephalitic complications in some of the patients(Nicoll et al., 2003). TSA and other HDAC inhibitors represent a new approach to treatment that appears to make the synapse more robust and resistant to the effects of Aβ. Our results suggest not only that TSA is capable of stopping memory degradation in the presence of Aβ accumulation but also that it may improve function in a brain that, as in the case of the 3-month-old double-transgenic mouse, has already lost function. Novel HDAC inhibitors in which the side effects have been minimized are being developed by the pharmaceutical industry. It remains to be seen if these newer inhibitors can readily enter the brain and if they are as effective as TSA.

Chapter 7

7 DISCUSSION

The work presented in this thesis is an investigation into the effect of WT PS1 and its mutant form on the transcriptional activity of CBP and p300 in AD, and of a possible therapeutic method for treating AD symptoms. In addition, the signalling pathways involved in the stimulus effect of CBP and p300 by WT PS1, but not by its mutant, were studied. Moreover, the specific regions of CBP activated by WT PS1 were localised, thus providing a further understanding of the molecular mechanism involved in the stimulation effect of WT PS1 stimulus effect. Additionally, the HAT activity of CBP was studied and an HDAC inhibitor was used in AD mice model in order to provide a better understanding of CBP role in AD.

Although in previous investigations CBP has been linked to AD, the role of PS1-CBP interaction in neurodegeneration, as is shown in the literature, is unclear and inconsistent. Moreover, no studies have directly tested CBP and p300 involvement in the disease. In addition, CBP/p300 have been shown to be associated with the human developmental disorder Rubinstein-Taybi syndrome, which is characterised by mental retardation. In view of this, we began to investigate the potential role of CBP in AD by studying its activity and the effects of manipulating its expression in cells containing wild type or mutant forms of PS1, which is mutated in the FAD.

The initial results presented in chapter 3 show that WT PS1 stimulates CBP activity over 2 fold. Therefore, an increase in CBP activity might be expected when stimulated with the presenilin 1 M146L mutant, however, no such increase

in activity was seen suggesting that the presenilin 1 M146L mutant cannot stimulate CBP activity directly. Moreover, the transfected Gal-CBP levels were shown not to change, suggesting the changes of CBP activity is as a result of changes in CBP enzymatic activity.

However, overall CBP activity could, in addition to changes in its enzymatic activity, also be altered by changes in its expression. We therefore measured endogenous levels of CBP and were able to show an increase in endogenous CBP when WT PS1 or its PS1-M146L mutant were transfected. However, CBP endogenous levels, when PS1-M146L was transfected, were significantly lower compared to the endogenous levels of CBP when WT PS1 was transfected. This suggests that, in addition to a decrease in CBP protein activity, decreased CBP levels might also be involved in the development of AD.

It is important to note that while wild type PS1 increases endogenous CBP level more than PS1- M146L mutant, this does not account for our Ga4-CBP results. Gal4-CBP activity changes cannot be explained by changes in Gal4-CBP level since Gal4-CBP levels stay the same during the luciferase experiments. Consequently effects on Gal4 promoter must be due to changes in CBP activity, enhancing its ability to activate transcription.

All the experiments discussed thus far use Gal4-CBP fusion proteins, which have in the past been criticised(Cardinaux et al., 2000). It has been suggested that the transcriptional activation function seen in Gal4-CBP is masked in the conformation of naïve CBP. Moreover, this conformation might not be

recapitulated precisely in the molecule that is targeted to a promoter through a heterologous DNA binding domain at its amino terminus. To overcome this observation, immunofluorescence of CBP was used to show the different effect of wild type and mutated PS1 on its cellular localization. The effect of WT and mutant PS1 on CBP localization were consistent with previous results showing stimulation of transcription by WT PS1, but not by its mutant form, thus confirming their validity.

As discussed in section 1.8.6, it was shown that PS1 may be involved in the processing and trafficking of membrane-bound proteins including the Notch receptor (Levitan and Greenwald, 1995; Wong et al., 1997), whereas PS1 protein deficiency and PS1 mutations block the endoproteolytic cleavage of Notch. In turn, Notch intracellular cytoplasmic domain (NICD), which is released from the Notch after endoproteolysis, translocates to the nucleus, where it is needed for modifying transcription of specific genes such as CBF1 (also known as RBP-Jκ). Interestingly, *Saura el al* (2004) found that the predicted CBP promoter contains a consensus recognition site for the transcription factor CBF1 through which the active form of the Notch intracellular domain exerts its transcriptional activation effects. This suggests that CBP is likely a downstream target gene of the Notch signalling pathway (Saura et al., 2004). This might explain how F11 cells expressing WT PS1 showed significantly higher levels of CBP than in F11 cells expressing PS1-M146L mutant or knockdown of PS1 (Fig 7.1).

An additional explanation to the increase in endogenous CBP levels, when WT PS1 is transfected, is that CBP localisation to the nucleus might protect CBP from

degradation. It would be intresting to see if CBP levels are regulated at the mRNA and/or protein level. We could use transcription inhibitors and test if CBP levels change with transfection of WT/mutant PS1. At the same time we could measure CBP total/nucleus levels to see if there are any changes.

Additionally, we were able to show generality with regards to the effect PS1 mutants have on CBP transcriptional activity. We were able to show that both N and C-terminal mutations of PS1 were not able to stimulate the transcriptional activation ability of CBP. Moreover, PS1 C-terminus mutations showed a decrease in CBP activity compared to control vector or PS1 N-terminus mutations. It is possible that the C- terminus mutants bind to a cellular protein, which regulates CBP activity, and inhibit this protein from being activated by endogenous PS1. In contrast, it is possible that N-terminus mutants do not bind to the cellular protein, which regulates CBP activity, and therefore do not inhibit its activation by endogenous PS1. N-terminus mutants therefore have no effect on CBP activity. It would be interesting to see if transfection of wild type PS1 would be able to produce a positive effect in the presence of N-terminal mutants, but not of the C-terminal mutants, as would be predicted by this model.

Since the activity of CBP was measured indirectly using constructs in which CBP was fused to the GAL-DBD, experiments were preformed to look at the effect of WT PS1 on the GAL-DBD portion of the protein alone to confirm that the results seen were valid. Indeed co-transfection of the GAL-DBD and a reporter construct showed no increase in reporter gene activity when cells were transfected with WT

PS1. This indicated that the transcriptional activity observed with Gal-CBP protein was due to the CBP portion of the construct.

Details of the pathway involved in the CBP up-regulation by WT PS1 came from experiments showing that inhibition of the PI 3-kinase, p38 MAP kinase and p42/p44 MAP kinase pathways, with the chemical inhibitors LY 294002, SB 203580 and PD 98059 respectively, blocked the increased activity of CBP induced by WT PS1. Theses results suggest that the up-regulation of CBP activity by WT PS1 was via these kinases pathways. Notably, chemical inhibitors are not generally very specific. Although they predominantly inhibit one kinase, they can inhibit other kinases. This lack of selectivity could be a problem and that it is worth following up these inhibitors, by transfecting dominant negative constructs of particular kinases, so as to get a more specific inhibitory effect.

It has been shown that three different main GluRs: AMPA, kainate and NMDA receptors activation are important for learning and memory (Bliss and Collingridge, 1993). The activation of these receptors leads to an activation of kinases, such as PKA, PKC, CaMKIV and MAPK. In addition to regulating CREB phosphorylation, all of these kinases have been shown to increase CBP activity. Interestingly, NMDA and MAPK activity has been shown to be reduced in activity in rodent with PS1 mutation (Ma et al., 2007; Wang et al., 2007). This might lead to a reduction in CBP activity and gene transcription, which are important for memory condensation (Fig 7.2).

Using small CBP fragments linked to Gal4 it was shown that activity of the CBP C-terminal region (amino acids 2173–2288) or CBP N-terminal regions (amino acids 1–460, 721–1679, 1678–1843) was increased over 2 and 1.5 fold respectively by WT PS1. However, the presenilin 1 M146L mutant form produced virtually no enhancement in stimulating activity of these regions, showing similar activity levels to the untreated cells. These results demonstrate that the presenilin 1 M146L mutant form, unlike wild-type Presenilin 1, has no ability to activate different regions of CBP. Notably, the decreased activity of the CBP region containing amino acids 721-1679 induced by presenilin 1 M146L mutant form was of particular interest since it contains the CBP acetyltransferase domain (Martinez-Balbas et al., 1998).

Since CBP and p300 are highly homologous proteins and are structurally similar, some of the experiments preformed for CBP were also done for p300. As in the case of CBP, p300 was shown to have enhanced activity when stimulated by WT PS1, but not by its mutant form. Interestingly, wild type Presenilin 1 and its M146L mutant form failed to increase the transcriptional activity of different regions of p300. This shows that the same stimulation can activate different regions of similar proteins. Also, multiple regions of the proteins may be involved both in the case of CBP and p300. In the case of CBP we demonstrated an effect when each of CBP regions is tested independently, whereas with p300 we might need to have all of p300 regions in the same construct to get a stimuli effect. It would be of interest to follow up the effect of WT PS1 on different regions of p300 by using point mutation in different regions.

The results thus far indicated a potential role for CBP and p300 in AD by responding to WT PS1 stimulation, but not to its FAD mutant. By using an antisense CBP construct, this hypothesis could be tested more directly. Use of the c-fos luciferase system confirmed this hypothesis by showing that the activation of the c-fos CRE promoter by WT PS1 is inhibited in cells co-transfected with antisense CBP.

CBP possesses HAT activity, which has previously been shown to increase their transcriptional activity(Martinez-Balbas et al., 1998). To identify the structural features of CBP involved in the stimuli response to WT PS1, a HAT mutation was used. The increased activity of the Gal4-CBP WT reporter gene induced by WT PS1 was not seen in cells transfected with the CBP HAT mutant construct. This demonstrates that WT PS1 stimuli effect is dependent on the HAT activity of CBP and it adds a new aspect to the mechanism by which the mutant of PS1 might be involved in AD. The HAT domain lies in the region 1200-1600 amino acids, inside the C-terminus which has been shown to increase promotor activity when stimulated by WT PS1. From these results, and work preformed by others(Ait-Si-Ali et al., 1999), it can be predicted that in the CBP full length protein, phosphorylation at the C-terminus will induce a conformational change of the CBP molecule that will allow the HAT domain to be more active.

The results so far suggest that it is the HAT activity of CBP, which is responding to the stimulating effect of WT PS1, but not to it PS1 mutant form. To further investigate this theory, an inhibitor of histone deacetylase activity, trichostatin A (TSA), was used to look at the effect of increased histone acetylation in an AD

mouse model. As predicted TSA was able to re-establish normal synaptic function in the APP/PS1 mouse by increasing potentiation in TSA treated APP/PS1 slices, which was far greater than in vehicle-treated APP/PS1 slices. Moreover, TSA was able to rescue normal memory in the APP/PS1 mouse as demonstrated by the improved contextual learning of APP/PS1 mouse treated with TSA.

To investigate the molecular mechanisms of TSA rescue, hippocampal neurons from APP/PS1 and WT mice were tested for their level of histone acetylation. As predicted, APP/PS1 mice displayed a decrease in histone 4 acetylation levels compared to WT mice, suggesting that AD might be a disease of epigenetic etiology. Remarkably, TSA administration to APP/PS1 and WT mice was able to rescue APP/PS1 mice reduced acetylation. This suggests that the rescuing of LTP and memory in APP/PS1 mice by HDAC inhibition is likely to be mediated, at least in part, by re-elevation in acetylation of histones H4.

Beneficial effects of histone acetylation in memory-related plasticity had previously been described both in WT animals and in animal models of human mental retardation(Alarcon et al., 2004; Korzus et al., 2004; Levenson et al., 2004). The importance of the work described in this thesis is that it shows, for the first time, that promoting histone acetylation restores learning, after synaptic and neuronal loss had already occurred. Together, these findings provide compelling evidence that increased histone acetylation can overcome the decrease of memory function seen in this AD mouse model.

The stimulation of the transcriptional activation ability of CBP by WT PS1, but not by its mutant form, does not correlate with recent studies reporting that the mutation in PS1 would caused enhanced levels of CBP which will promote neurodegeneration(Marambaud et al., 2003; Teo et al., 2005). However, our work correlates with a previous study which showed reductions in CBP levels and in CRE-dependent gene expression in the PS 1 and 2 conditional double knockout mice(Saura et al., 2004) hence suggesting that decreased synthesis/activity of CBP may be important in the neurodegeneration observed in AD. Moreover, CBP-dependent gene expression has been demonstrated to play an important role in the consolidation of long-term forms of synaptic plasticity and memory. In our study we found a consistent reduction in CBP/p300 activity, including a specific reduction in CBP HAT activity that might have caused a reduction in histone 4 acetylation. Since CBP is an essential cofactor for the formation of long-term memory, diminished CBP activity represents the likely basis for the global reduction of memory consolidation in APP/PS1 mutant mice. While the basis for the discrepancy between the two studies is unclear, it may reflect differences in methodology or experimental system (i.e., cell culture versus adult brain).

The above data suggests that CBP and its HAT activity are essential for enhancing transcription in vitro following WT PS1 stimulation. It would be interesting to perform further experiments that will to determine CBP levels and HAT activity in mouse model of AD. In addition, it would be of value to test for acetylation levels in other histone than histone 4. Moreover, it is possible that the reduction in histone 4 acetylation rises from an increase in HDAC activity and not a reduction of HAT activity. Thus, it would be important to determine HDAC activity in

App/PS1 mice and compare it to WT mice. Furthermore, it is important determine whether HDAC inhibitors, other than TSA, will be able to re-establish synaptic function and normal memory in the APP/PS1 mouse.

Recent studies indicate that there are alterations in immune cell function in AD, and a role for PS1 in immune regulation has been suggested recently. Thus, PS1-mutant M146V knockin mice have been shown to have defects in its immune function(Morgan et al., 2007). Therefore, it would be of interest to see if a similar decrease in histone acetylation can be found in T lymphocytes. Lastly, it would be worth testing if the same histone acetylation difference appears in human brains of Alzheimer's patients, which represents a more accurate model for the disease.

The work reported in this thesis leads to many interesting areas of research for future work, and is the first direct evidence for the involvement of CBP and p300 in AD. It introduces the possibility of novel therapies targeting CBP and p300 and in particular histone acetylation, it also adds some understanding to the conflicting literature obtained to date as to how CBP and p300 are involved in AD.

Figure 7.1 Notch-PS1 pathway

Ligand-receptor interactions lead to γ-secretase cleavage of Notch and release of the intracellular domain (NICD). This domain enters the nucleus and together with a complex including CBF1 and MAML promotes the transcription of targets such as the Hes, Herp and CBP genes (figure adapted from (Yoon and Gaiano, 2005)).

Figure 7.2 Signal transduction mechanism for long-term memory and its potential deregulation by PS1 mutant form.

A variety of signal pathways contribute to creation and reinforcement of memory in the hippocampal neurons, which lead to protein synthesis. Mutated forms of presenilin that are associated with Alzheimer's disease are thought to accelerate the production of toxic amyloid plaques. These presenilin mutants also interrupt NMDA and MAPK activation. This might lead to a decrease of CBP activation, which is downstream from NMDA and MAPK. Therefore, as well as provoking amyloid-related neurotoxicity, presenilin mutations might cause Alzheimer's disease by inappropriately deactivating genes associated with neuronal function and memory formation (figure adapted from (Voglis and Tavernarakis, 2006)).

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